26834

National Library of Canada | Bibliothèque nationale du Canada

CANADIAN THESES ON MICROFICHE | *THÈSES CANADIENNES SUR MICROFICHE*

NAME OF AUTHOR/*NOM DE L'AUTEUR* SHEILA GRACE MCKENZIE

TITLE OF THESIS/*TITRE DE LA THÈSE* ON THE MECHANISM OF ACTION OF ADENOSINE

UNIVERSITY/*UNIVERSITÉ* ALBERTA

DEGREE FOR WHICH THESIS WAS PRESENTED/ *GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* PH D

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1975

NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* HANS-PETER BÄR

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

DATED/*DATÉ* Sept 25, 1975 SIGNED/*SIGNÉ* S. G. McKenzie

PERMANENT ADDRESS/*RÉSIDENCE FIXÉ* 31 ARDS DRIVE
GLASGOW G52 1TN
SCOTLAND

NL-91 (3-74)

THE UNIVERSITY OF ALBERTA

ON THE MECHANISM OF ACTION OF ADENOSINE

by

SHEILA GRACE McKENZIE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1975

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ON THE MECHANISM OF ACTION OF ADENOSINE submitted by SHEILA GRACE MCKENZIE in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

.....
Supervisor

.....

.....

.....

.....

.....
External Examiner

Date September 5, 1975

ABSTRACT

Renewed interest in the mechanism of action of adenosine and its nucleotides has been generated by recent evidence of both humoural and neural physiological roles for these substances. Adenosine and the adenine nucleotides have also been demonstrated to elevate cyclic AMP levels in certain tissues and cultured cell lines. Cyclic nucleotides may be codeterminants of smooth muscle tone and, in particular, relaxation by beta-adrenergic agonists has been associated with enhanced intracellular cyclic AMP levels. The hypothesis was tested that adenosine and its nucleotides relax smooth muscle by elevating tissue cyclic AMP content in a manner analogous to that proposed for the beta-adrenergic catecholamines.

In the isolated longitudinal muscle of the rabbit intestine, adenosine and its nucleotides were equipotent in inhibiting spontaneous isometric contractions and dose-dependent responses were observed between 0.1 and 100uM. Although progressively diminished by storage at 4°C, responses were unaffected by local anaesthetics, guanethidine and reserpine, indicating that the drugs act directly on smooth muscle. Responses to adenosine and ATP were similarly unaffected by inhibitors of nucleoside transport, adenosine deaminase or 5'-nucleotidase or by

imidazole or 1-methyl,3-isobutylxanthine, but they were antagonised by theophylline. The antagonism could be overcome by higher doses of agonist. Autoinhibition was observed with cumulative doses of adenosine or ATP and was mutually effective. Results were consistent with a common extracellular site of action for adenosine and its nucleotides.

By investigation of a small series of available adenosine analogues, the moieties necessary for adenosine-like activity in the rabbit intestine were found to be a primary or secondary amino group at the N^6 position and hydroxyl groups at the 2'- and 3'-positions. 8-bromoadenosine was inactive. No antagonistic activity was observed with inactive analogues.

Adenosine, at or above 10uM, inhibited adenylate cyclase prepared from a variety of mammalian tissues. In rat brain and rabbit heart the inhibition was shown to be non-competitive with respect to ATP. Among the series of adenosine analogues, no correlation was found between analogues which relaxed longitudinal muscle and those which inhibited adenylate cyclase prepared from that tissue. It is concluded that adenosine-induced inhibition of cyclase, although apparently a general phenomenon of mammalian systems, need not participate in pharmacological

responses to adenosine.

No change in tissue cyclic AMP content, as assayed by a protein binding method, was found in response to 1-1000uM adenosine or to 100-1000uM ATP. The phosphodiesterase inhibitors theophylline and 1-methyl,3-isobutylxanthine did not modify this outcome, although theophylline antagonised adenosine responses and methyl-isobutylxanthine caused relaxation as well as a small increase in cyclic AMP levels when given alone. Significant increases in tissue cyclic AMP were also observed with isoproterenol and epinephrine. No support was therefore obtained for the hypothesis that adenosine relaxes smooth muscle by elevating tissue cyclic AMP.

ACKNOWLEDGMENT

To my supervisor, Dr. H.-P. Baer, I offer my sincere thanks for his advice, encouragement and patience throughout the course of this undertaking. I also wish to thank Gilles Lauzon, Sudha Kulshrestha, Laurie Simonson and all other members of the Department of Pharmacology who contributed to the friendly and co-operative atmosphere in which the work was carried out. Production of this thesis was greatly facilitated by the graphics of Fred Loeffler and Ken Burt and by the electron microscopy of Gus Duchon. I am grateful to the Medical Research Council of Canada for personal financial support and to the Alberta Heart Foundation for funding the research.

TABLE OF CONTENTS

CHAPTER		PAGE
1. INTRODUCTION		1
1.1.	The pharmacology of adenosine	1
1.1.1.	Membrane permeability to adenosine and nucleotides	3
1.1.2.	Adenosine antagonists	6
1.1.2.1.	Methylxanthines	7
1.1.3.	Drugs potentiating adenosine responses ..	11
1.1.4.	Evidence of more than one adenosine receptor	13
1.2.	Postulated physiological roles for adenosine and nucleotides	15
1.2.1.	Humoural	16
1.2.2.	Neural	20
1.3.	Postulated mechanisms of action of adenosine and nucleotides	22
1.4.	Effects of adenosine on cyclic AMP levels of intact cells or tissue slices	26
1.5.	Effects of adenosine on enzymes related to cyclic AMP	29
1.5.1.	Adenylate cyclase	29
1.5.2.	Phosphodiesterase	30
1.5.3.	Protein kinase	30
1.6.	The postulated role of cyclic AMP in smooth muscle relaxation	31
1.7.	Objectives of this research	34

2. MATERIALS AND METHODS 36

2.1. Longitudinal muscle of the rabbit intestine 36

2.1.1. Preparation of the tissue 36

2.1.2. Measurement of relaxant response 39

2.1.3. Measurement of tissue cyclic AMP levels 42

2.1.3.1. Tissue sampling 42

2.1.3.2. Homogenisation 44

2.1.3.3. Purification 46

2.1.3.4. Assay of cyclic AMP 49

2.1.3.5. Expression of results 59

2.1.4. Preparation of adenylate cyclase 59

2.2. Preparation and assay of adenylate cyclase from various tissues 64

2.2.1. Preparation of adenylate cyclases 64

2.2.2. Assay of adenylate cyclase activity 65

2.3. Chemicals 66

3. RESULTS AND DISCUSSION 68

3.1. Relaxant responses 68

3.1.1. Adenosine and analogues 69

3.1.1.1. The adenosine response 69

3.1.1.2. Effects of cold storage 73

3.1.1.3. Investigation of neural involvement in the adenosine response 74

3.1.1.4. Influence of drugs on the adenosine response 76

3.1.1.5. Adenosine analogues 77

3.1.1.5.1.	Responses to analogues in longitudinal muscle	77
3.1.1.5.2.	Comparison of active analogues	80
3.1.1.6.	Antagonism of adenosine responses	86
3.1.2.	Sympathomimetics	89
3.1.3.	Discussion of relaxant responses	90
3.2.	Effects of adenosine on adenylate cyclase	102
3.2.1.	Characteristics and kinetics of adenosine inhibition	102
3.2.1.1.	Time dependence and reversibility	105
3.2.1.2.	Kinetics	108
3.2.2.	Effect of adenosine analogues on adenylate cyclase	111
3.2.3.	Discussion of adenosine inhibition of adenylate cyclase	117
3.3.	Tissue cyclic AMP levels and associated relaxant responses	127
3.3.1.	Variation	127
3.3.2.	Sensitivity testing	130
3.3.3.	Effect of adenosine on cyclic AMP levels	141
3.3.4.	Discussion of cyclic AMP measurements ...	151
3.4.	Conclusions	162
BIBLIOGRAPHY		166
APPENDIX		183

LIST OF TABLES

TABLE		PAGE
1.	Specific activity and yield of fractions during preparation of phosphodiesterase	55
2.	Cyclic AMP breakdown in phosphodiesterase-treated samples	58
3.	Effect of 1mM adenosine on adenylate cyclases	103
4.	Kinetic constants from programme HYPER: rat brain	110
5.	Kinetic constants from programme NONCOMP: rat brain	112
6.	Kinetic constants from HYPER and NONCOMP: rabbit heart	113
7.	Effect of analogues on longitudinal muscle adenylate cyclase	114
8.	Multiple cyclic AMP measurements from individual animals	129
9.	Relaxation and change in cyclic AMP levels in response to 1uM isoproterenol, epinephrine and adenosine	132
10.	Tissue cyclic AMP levels 60s after 0.05uM isoproterenol ± 0.1mM theophylline	135
11.	Analysis of variance table: 0.05uM isoproterenol versus 0.1mM theophylline	137
12.	Tissue cyclic AMP levels 60s after 0.05uM epinephrine ± 0.1mM theophylline	139
13.	Analysis of variance table: 0.05uM epinephrine versus 0.1mM theophylline	140
14.	Tissue cyclic AMP levels 60s after 10uM adenosine ± 0.1mM theophylline	142
15.	Analysis of variance table: 10uM adenosine versus 0.1mM theophylline	143

16. Tissue cyclic AMP levels 60s after 100uM adenosine ± 10uM methylisobutylxanthine 145

17. Analysis of variance table: 100uM adenosine versus 10uM methylisobutylxanthine 146

18. One-way analysis of variance with six tissues per group 149

19. Analysis of variance table: adenosine or ATP at 0.1 and 1mM versus animals 150

LIST OF FIGURES

FIGURE		PAGE
1.	Electron micrograph of the experimental tissue	38
2.	Apparatus for measurement of relaxant responses and rapid tissue sampling	40
3.	Responses to adenosine, epinephrine and isoproterenol	43
4.	Flow diagram of preparatory procedure for measurement of tissue cyclic AMP levels	45
5.	Elution profile from Dowex of ^{3}H-cyclic AMP, ATP and 5'-AMP	47
6.	Standard curve from cyclic AMP assay	50
7.	Preparation of phosphodiesterase, Part I	53
8.	Preparation of phosphodiesterase, Part II	54
9.	Cyclic AMP formation and ATP breakdown by an adenylate cyclase preparation from longitudinal muscle	63
10.	Dose-response curves to adenosine	70
11.	Autoinhibition of the adenosine response	72
12.	Adenosine analogues employed in this study	78
13.	Dose-response curves to adenosine and ATP	81
14.	Cumulative responses to alpha,beta-methylene-ADP	85
15.	Structures of adenosine, theophylline and methylisobutylxanthine	88
16.	Dose-effect curve of adenosine on adenylate cyclase from rat brain	104
17.	Time dependence and reversibility of adenosine-induced inhibition of cyclase	106

18. Reversal of adenosine-induced inhibition of cyclase 107

19. Double reciprocal plot of rate of cyclase activity versus substrate concentration at three concentrations of adenosine 109

20. Non-additivity of the effects of adenosine and 3'-deoxyadenosine on cyclase from rat brain 116

21. Additivity of the effects of adenosine and theophylline on cyclase from guinea-pig lung .. 118

CHAPTER 1

INTRODUCTION

1.1. The pharmacology of adenosine.

In 1929 Drury & Szent-Gyorgyi identified as adenylic acid the active principle in extracts from mammalian hearts which caused depression of blood pressure and heart rate and increased coronary flow in anaesthetised animals. Similar responses were elicited by injection of AMP or adenosine. Since that date, pharmacological responses to adenosine and its nucleotides have been described in many systems and by many authors. Generally these substances are smooth muscle effectors, relaxing most vascular, mammalian intestinal and bronchial muscle and exciting a few systems, including renal and lung vasculature (Drury, 1936), urinary bladder, vas deferens and intestine of lower vertebrates (Burnstock, 1972). Intermediate responses involving both inhibitory and excitatory phases are observed with both adenosine and its nucleotides in the gut and uterus of some species (Burnstock, 1972 and Burnstock et al., 1972b). Adenosine, as opposed to its nucleotides, will not contract urinary bladder (Burnstock et al., 1972a), indicating that the nucleoside does not share all the effects of its nucleotides.

Other pharmacological effects of adenosine which have been discovered over the past fifty years are negative chronotropic and inotropic responses in the heart (Drury & Szent-Gyorgyi, 1929), observed at doses higher than necessary to increase coronary flow (Schoendorf et al., 1969), inhibition of thrombocyte aggregation (Born et al., 1964) and an insulin-like effect in adipose tissue (Dole, 1961 and 1962). The ability of adenosine and its nucleotides to elevate cyclic AMP levels in slices of brain tissue and other cultured cell types is described in Section 1.4.

Although adenosine and its nucleotides elicit both inhibitory and excitatory responses in smooth muscle, it has so far proved impossible to distinguish betweeen the receptors responsible for these two types of action. The same order of potency of the substances (ATP ≥ ADP > AMP ≥ adenosine) has been observed by most workers for both types of responses, but Burnstock (1972) remarked that the true relative potencies of these analogues may be obscured by the rapid uptake of adenosine into tissues. Relative potencies may also be influenced by rapid metabolism of the nucleotides by extracellular phosphorolytic enzymes. Burnstock et al. (1975), however, reported that the 'rebound contraction' following inhibition of intestinal

tone by ATP, which is observed in many species, is blocked by indomethacine. They concluded that this rebound phenomenon, which is observed after ATP washout, is mediated by a prostaglandin. The major obstacle in the elucidation of the mechanism or mechanisms of adenosine action is the current lack of an effective competitive antagonist for any response to adenosine or adenine nucleotides.

1.1.1. Membrane permeability to adenosine and nucleotides.

Kuebler & Bretschneider (1963) demonstrated that, although uptake of 0.5-5mM adenosine into erythrocytes followed Michaelis-Menton kinetics and was temperature dependent, transport was not inhibited by sodium fluoride (a potent inhibitor of ATPase) and possessed a low activation energy similar to that for facilitated diffusion. Since intracellularly transported adenosine was rapidly phosphorylated or deaminated, exchange diffusion could not be demonstrated, but transport was inhibited by inosine. Kuebler & Bretschneider (1963) concluded that adenosine and other nucleosides permeate erythrocyte membranes by facilitated diffusion. Oliver & Paterson (1971) demonstrated that the specificity of the

erythrocyte transport mechanism encompassed both purine and pyrimidine nucleosides and that uridine efflux was enhanced by extracellular adenosine. The mechanism was inhibited by dipyridamole (Kuebler & Bretschneider, 1964) and by nitrobenzylthioguancsine (Brown & Paterson, 1971).

These drugs also inhibit the transport mechanism in dog heart (Olsson, 1972), which has much stricter binding requirements resembling those for adenosine-like activity in the myocardium (Olsson et al., 1973). Dipyridamole was found by Hopkins & Goldie (1971) to block adenosine uptake into guinea-pig heart but not into rat heart. To account for this species difference and in view of the fact that dipyridamole selectively reduced adenosine phosphorylation in slices of guinea-pig heart while having no effect on adenosine metabolism (phosphorylation or deamination) in rat heart slices, Hopkins & Goldie proposed that dipyridamole inhibits a membrane-bound adenosine kinase which is not present in rat heart and which is responsible for most of the accumulation of radioactivity when guinea-pig heart is exposed to radioactive adenosine. Hulme & Weston (1974b) similarly concluded that dipyridamole, hexobendine and lidoflazine inhibit adenosine phosphorylation in the longitudinal muscle of the rabbit intestine and that, by so doing, these drugs allow accumulation of intracellular adenosine, reduce the

transmembrane concentration gradient and prevent passive influx of extracellular adenosine. Plagemann (1971), Roos & Pfleger (1972) and Schrader et al. (1972), however, performed more careful studies which all indicated that, whereas at low extracellular concentrations adenosine is transported across biological membranes by a carrier-mediated system, above 0.1mM a component of passive diffusion begins to predominate. The metabolic fate of adenosine depends upon its intracellular concentration, the Km for adenosine kinase being lower than that for either deaminase or transport (Schrader et al., 1972 and Olsson et al., 1972). Since each of these groups of workers found that dipyridamole competitively inhibited carrier-mediated diffusion and not phosphorylation of adenosine by broken-cell preparations, they concluded that transport is the rate-limiting step in the phosphorylation reaction and that transport inhibitors therefore appear to inhibit phosphorylation selectively. Adenosine was substantially deaminated only if the extracellular concentration was sufficiently high to allow simple diffusion to raise intracellular adenosine towards the Km for deaminase.

Adenosine nucleotides are not believed to permeate cell membranes *per se*, but seem to be transported as adenosine in mouse ascites tumour cells (Williams & le

Page, 1958), myocardium (Hoffmann & Okita, 1965 and Hopkins, 1973a) and guinea-pig taenia coli (Lynch, see Burnstock, 1972).

1.1.2. Adenosine antagonists.

Responses to adenosine and its nucleotides in several tissues are antagonised by a number of antimalarial drugs, including mepacrine, quinine and quinidine (Madinaveitia & Raventos, 1949). Adenosine has been postulated to play a role in the pathogenesis of malaria (Onabanjo & Maegraith, 1970) and a parallelism between antimalarial activity and antagonism of adenosine responses was reported by the former workers. The cinchona alkaloids and their derivatives are not, however, specific adenosine antagonists, since Burnstock et al. (1970) reported that they antagonise catecholamine-induced responses in guinea-pig taenia coli at doses lower than those required to antagonise responses to ATP. Similar non-specific antagonism is observed with high doses of phentolamine and imidazole.

Burnstock (1972), in discussing the conclusion of Arulappu (see Burnstock, 1972) that quinidine blocks relaxant responses to adenosine in the uterus while

phenothiazines and dibenzazepines block contractile responses in the same tissue, argued that these drugs do not selectively antagonise inhibitory or excitatory responses in gut or bladder. 2-2'-pyridylisatogen has recently been reported to be a specific, non-competitive antagonist for ATP responses in guinea-pig caecum (Spedding et al., 1975).

1.1.2.1. Methylxanthines.

The methylxanthines which, as purines, bear certain structural resemblances to adenosine, are also adenosine antagonists. Theophylline, which appears to be the most potent of the methylxanthines, antagonises the effects of adenosine and ATP on coronary flow (Afonso, 1970; Schaumann et al., 1970 and Wadsworth, 1972), cardiac rate and force (Ther et al., 1957; Afonso & O'Brien, 1970 and Schaumann et al., 1970), systemic blood pressure (Afonso & O'Brien, 1970 and Schaumann et al., 1970), platelet aggregation (Schaumann et al., 1970 and Mills & Smith, 1971) and cyclic AMP accumulation in brain slices (Sattin & Rall, 1970). The influence of theophylline on adenosine responses in isolated smooth muscle has, however, not been widely reported. The distinguishing feature of theophylline is that, at doses which antagonise adenosine

responses, no antagonism of responses to catecholamines or acetylcholine occurs; on the contrary, catecholamine responses are frequently potentiated. Only Bowman & Hall (1970) reported that, in rabbit intestine, theophylline did not distinguish between catecholamine- and adenosine- or ATP-induced relaxation. The methylxanthines also possess other pharmacological properties which may contribute to their inhibition of adenosine and nucleotide responses and which restrict their usefulness for selective purposes.

Inhibition of phosphodiesterase has been suggested as a mechanism whereby theophylline antagonises adenosine responses in the myocardium (Schaumann et al., 1970), but no supportive evidence was presented by these workers. On the other hand, the opposing effects of adenosine or analogues and theophylline on lipolysis may be partly due to divergent effects on cyclic AMP in adipose tissue (Davies, 1968; Dietmann & Juhran, 1971 and Fain, 1973).

Theophylline antagonises intracellular transport of adenosine and other nucleosides (Huang & Daly, 1974; Plagemann & Sheppard, 1974 and Woo et al., 1974). Huang & Daly, however, found that this could not account for the inhibition by theophylline of adenosine-induced elevation of cyclic AMP in brain slices. Since adenosine transport

inhibitors generally potentiate adenosine responses (see Section 1.1.3), Woo et al. (1974) suggested that, due to its purine structure, theophylline binds to both the sites of action of nucleosides and to their transport sites.

Despite evidence that caffeine promotes transmembrane calcium exchange in the heart and that adenosine produces the opposite effect (Nayler, 1963; Grossman & Furchgott, 1964 and Guthrie & Nayler, 1967), Chiba et al. (1973) were unable to demonstrate any effect of high calcium on adenosine responses in the sinus node. Grossman & Furchgott (1964) cautioned that the rate of calcium exchange may reflect, rather than initiate, changes in myocardial contractility, but Nayler & Hasker (1966) demonstrated that caffeine releases calcium bound to subcellular fractions of cardiac muscle. De Gubareff & Sleator (1965) suggested that antagonism between caffeine and adenosine on myocardial contractility is exerted at a critical calcium-binding site controlling the availability of calcium to the contractile machinery. Somlyo & Somlyo (1968) suggested that caffeine also mobilises calcium in vascular smooth muscle, causing an initial contraction which is superceeded by cyclic AMP-mediated relaxation. Although effects of theophylline on calcium movements have not been intensively studied, evidence that the positive inotropic effect of theophylline is more likely to be due

to mobilisation of intracellular calcium or enhanced calcium influx than to inhibition of cyclic nucleotide phosphodiesterase was presented by McNeill et al. (1969). Bellemann & Scholz (1975) found the inotropic effect of theophylline to be dependent on extracellular calcium and furthermore dissociated the pharmacological effect from uptake of the drug. This suggests that theophylline acts at the plasma membrane, possibly, but not necessarily, to modify directly transmembrane calcium flux. It is therefore possible that both caffeine and theophylline interact with adenosine on the availability of cytoplasmic calcium in the heart and conceivably also in smooth muscle.

In contrast to the above evidence, theophylline and other methylxanthines selectively antagonise the accumulation of cyclic AMP induced by adenosine in brain slices (Sattin & Rall, 1970; Huang et al., 1972 and Huang & Daly, 1974), mouse neuroblastoma cells (Blume et al., 1973), astrocytoma cells (Clark et al., 1974) isolated bone cells (Peck et al., 1974) and thrombocytes (Mills & Smith, 1971). Haslam & Lynham (1972) have demonstrated inhibition by theophylline of an adenosine-sensitive adenylate cyclase in thrombocytes. The conclusion drawn by these and other workers is that theophylline antagonises the action of adenosine at a specific

extracellular receptor in close association with adenylate cyclase. Clark & Gross (1974) concluded that in astrocytoma cells the antagonism at this site is competitive. Thus, methylxanthines are specific antagonists of the effects of adenosine in several related systems, theophylline being the most potent. Despite the complicated action of theophylline which appears to proceed by several mechanisms and its relatively poor selectivity in some systems, it would appear to be the best choice at this time for studies of the antagonism of the response to adenosine, especially in systems where an effect of adenosine on adenylate cyclase is postulated.

1.1.3. Drugs potentiating adenosine responses.

Although Deuticke & Gerlach (1966) found a correlation between the ability of dipyridamole (and a series of its derivatives) to inhibit adenosine deaminase and to potentiate adenosine-induced coronary dilation, a closer dose relationship has been found between potentiation of responses and inhibition of adenosine uptake by tissues (Pfleger et al., 1969 and Kuebler et al., 1970), as first suggested by Kuebler & Bretschneider (1964). Drugs which inhibit adenosine transport into cells are widely reported to potentiate responses to

adenosine and its nucleotides. These drugs are most effective in intact animals where uptake of adenosine into various organs is the primary means of inactivation of adenosine and nucleotides. The significance of rapid uptake of adenosine into the lung of some species and of inhibition of this process by dipyridamole and hexobendine has been emphasised by Pfleger & Schoendorf (1969), Kolassa et al. (1970) and Afonso & O'Brien (1971). Pfleger et al. (1969) also demonstrated potentiation of adenosine-induced cardiac depression by inosine via a similar mechanism. Lidoflazine, however, appears to possess an additional potentiatory action (Hopkins, 1973b), possibly exerted on the smooth muscle itself (Afonso et al., 1968). In studies using isolated tissues, contradictory results have been obtained with transport inhibitors (Stafford, 1966; Hopkins & Goldie, 1971; Satchell et al., 1972 and Hulme & Weston, 1974a), possibly due to the interplay between extracellular phosphorolytic enzymes, adenosine concentration, total extracellular adenosine, mechanism and rate of adenosine uptake by the tissue and its metabolic fate.

Ouabain, an inhibitor of sodium-potassium activated ATPase, potentiates responses to adenosine in guinea-pig heart (Rand et al., 1955) but also potentiates acetylcholine responses (Rand & Stafford, 1957). Since

Axelsson & Holmberg (1969) found that g-strophanthin did not influence ATP-induced relaxation of the guinea-pig taenia coli, inhibition of ATPase may not be regarded as a selective means of modifying responses to adenosine or ATP.

1.1.4. Evidence of more than one adenosine receptor.

Since adenosine readily permeates cell membranes (see Section 1.1.1) and therefore has the potential to interfere in many biochemical reactions, its site of action in all tissues is certainly not upon a classical pharmacological 'receptor'. Nevertheless, the facts that the nucleotides, which are unlikely to enter cells per se, are frequently more potent than adenosine and also that inhibitors of adenosine transport do not antagonise responses but on the contrary frequently potentiate them, indicate that in the cardiovascular system, in smooth muscle and in the brain an extracellular site of action is most likely. Among these systems, attempts are being made to characterise the binding requirements at specific 'receptor' sites. Despite the lack of good pharmacological tools, evidence of the existence of more than one adenosine receptor has lain latent since Wedd (1931) reported that whereas 3'-AMP, like 5'-AMP, is an

effective coronary vasodilator, 3'-AMP is a relatively weak cardiodepressant. Similar findings were reported by James (1965). Finally, Einstein et al. (1972), on the basis of divergent effects of 2-alkylthio- as opposed to 2-halogeno-adenosines and adenosine itself, postulated the existence of 'cardiac' and 'vascular smooth muscle' receptors which differ in binding requirements.

Born et al. (1965) has found the relative potencies of a series of adenosine analogues to be equivalent as inhibitors of thrombocyte aggregration and as vasodilators in man, but unfortunately his series did not encompass the 2-alkylthio-adenosines, the group found to be ineffective on cardiac receptors by Einstein et al. Sattin & Rall (1970) and Huang et al. (1972) provided evidence that the binding requirements in brain are essentially similar to those in smooth muscle, namely that the amino group in the 6-position and an intact ribose ring are essential for activity. The effects of 2-alkylthioadenosines in brain have not yet been reported. In all these systems, inosine and adenine are virtually inactive.

Further possible differences in binding requirements were delineated by Leslie et al. (1973) who concluded that, whereas analogues with a modified purine ring are more effective in relaxing guinea-pig ileum than they are

in relaxing guinea-pig aorta, 2'-deoxyadenosine relaxes vascular smooth muscle but is inactive in the intestine. It is as yet unknown whether any of these receptors which demonstrate different binding requirements for activation reflect pharmacologically and biochemically distinct entities, perhaps analogous to the alpha and beta receptors for the catecholamines, or whether they merely represent a range of subgroups as is observed within the category of beta-adrenergic receptors.

1.2. Postulated physiological roles for adenosine and nucleotides.

Ever since pharmacological responses to adenosine and its nucleotides were first observed, considerable speculation has taken place concerning possible physiological roles for these ubiquitous substances. In an early revue, Drury (1936) discussed the possibility that adenosine is involved in reactive hyperaemia and vasodilation accompanying muscular activity (autoregulation), among other possibilities. Almost thirty years later Berne (1963) presented evidence that adenosine is a metabolic product during hypoxia and could be responsible for autoregulation of coronary flow. Further interest in the pharmacological effects of

adenosine and its nucleotides was initiated in 1970 when Burnstock et al. postulated that ATP or a related adenine nucleotide is the transmitter at non-adrenergic inhibitory nerve terminals in the gut. According to these two hypotheses, adenosine and/or ATP resembles epinephrine/noradrenaline in possessing both humoural and neural physiological roles. The evidence for each of these hypotheses is considered independently below.

1.2.1. Humoural.

Berne proposed in 1963 that adenosine is the mediator of coronary autoregulation on the basis of the observation that breakdown products of adenosine appear in the perfusate from hypoxic but not from normal hearts. Although these metabolites (inosine and hypoxanthine) are inactive, the quantity of the parent compound, adenosine, was calculated to be sufficient to account for the observed coronary dilation. Since adenosine, but not its nucleotides, freely permeates myocardial cells (Hoffman & Okita, 1965), adenosine was proposed to be the humoural agent responsible. In the same year Gerlach et al. (1963) elucidated the catabolic pathway for adenine nucleotides in heart and also concluded that sufficient adenosine is produced in myocardial cells during anoxia to account for

considerable coronary vasodilation after diffusion of the material into the vasculature. As further support for the hypothesis, Richman & Wyborny (1964) and Katori & Berne (1966) were able to demonstrate adenosine itself in the perfusion fluid of hypoxic but not normal hearts, in the presence of 8-azaguanine, an inhibitor of adenosine deaminase. Adenosine could be detected in the absence of a deaminase inhibitor only subsequent to complete ischaemia or coronary occlusion (Imai et al., 1964; Rubio et al., 1969 and Olsson,1970).

Since adenosine could also be detected in venous blood from skeletal muscle after a period of ischaemic contraction (Berne et al., 1971 and Dobson et al., 1971), adenosine was also postulated to mediate autoregulation in that tissue. Tominaga et al. (1973) found that venous effluent from skeletal muscle undergoing postischaemic contraction, postexercise hyperaemia or reactive hyperaemia, could produce renal vasoconstriction. Since adenosine and AMP are known to induce this opposing response in renal vasculature, their results are also consistent with the hypothesis of involvement of adenosine in autoregulation.

Further support for the 'adenosine' hypothesis has been sought by several workers but conflicting reports

have also appeared. Moir & Downs (1972) found even high concentrations of adenosine and ATP to be incapable of increasing coronary flow to the extent observed in reactive hyperaemia and concluded that these substances are not the sole mediators of this response. Since reactive hyperaemia reflects pathological as opposed to physiological regulation of blood flow, it is likely that several vasodilatory substances, as well as noradrenaline (Wollenberger et al., 1969), are released into the blood stream following coronary occlusion. Thus this observation does not preclude the possibility that adenosine is the normal physiological mediator of coronary vasodilation.

Theophylline, a potent adenosine antagonist, does not influence either reactive hyperaemia (Juhran & Dietmann, 1970 and Bittar & Pauly, 1971) or the autoregulatory response to hypoxia (Afonso et al., 1972 and Wadsworth, 1972) in the heart. Wadsworth (1972) reported, however, that theophylline reduces the duration of reactive hyperaemia in cat heart. If it were not for the fact that theophylline is capable of causing a number of pharmacological responses, these studies would constitute a rather definitive objection to the adenosine hypothesis.

In contrast, dipyridamole potentiates cardiac

reactive hyperaemia (Miura et al., 1967; Bittar & Pauly, 1970 and 1971) and is reported to potentiate exercise hyperaemia in the dog hind limb (Miura et al., 1967). Bell (1974), however, found that dipyridamole also potentiates vasodilatory responses to glyceryl trinitrate in the guinea-pig parametrial artery, suggesting that the specificity of dipyridamole for adenosine responses is suspect. Although Bittar & Pauly (1971) reported that lidoflazine does not potentiate myocardial reactive hyperaemia, Raberger et al. (1973b) found hexobendine to potentiate responses to both short-lasting, generalised hypoxia and single intracoronary injection of adenosine to a similar extent. In summary, although adenosine may be formed in cardiac and skeletal muscle during hypoxia in quantities sufficient to cause vasodilation, sound pharmacological evidence of an autoregulatory role for adenosine is lacking. Possibly, the extreme anoxia imposed under most experimental conditions causes release of pathological vasodilatory metabolites and other substances which obscure the effects of the physiological mediator.

1.2.2. Neural.

The hypothesis that ATP or a related adenine nucleotide is the transmitter substance at non-adrenergic inhibitory nerve terminals in the gut was proposed by Burnstock et al. (1970), on the basis that:

1) enzymes for the synthesis and degradation of the postulated transmitter are present in the tissue.

2) nerve stimulation caused release of ATP, ADP and AMP and/or adenosine and inosine into the medium, of which ATP and ADP were the most potent smooth muscle relaxants.

3) ATP mimicked the response to nerve stimulation.

4) quinidine antagonised responses to ATP and to nerve stimulation at similar concentrations.

5) responses to nerve stimulation were depressed when tachyphylaxis to ATP had been induced in the tissue.

Since then, considerable support for the hypothesis has been obtained by several workers who have demonstrated the ability of ATP to mimic both non-adrenergic inhibitory and non-cholinergic excitatory nerve stimulation in several tissues of various vertebrate species (Dumsday, 1971; Burnstock et al., 1972a and 1972b; Nakanishi & Takeda, 1972, Coleman & Levy, 1974 and Kalsner, 1974). The subject has been reviewed by Burnstock (1972), where specific uptake of adenosine into nerve terminals and

storage as ATP (Su et al., 1971) and potentiation of the response to nerve stimulation by dipyridamole and hexobendine (Satchell et al., 1972) are described together with other supportive evidence.

Despite this predominantly circumstantial evidence in favour of the hypothesis, doubt has been shed on the specificity of uptake and release of labelled nucleotides by Auerbach's plexus (Kuchii et al., 1973a, 1973b and 1974) and on the effectiveness of tachyphylaxis to ATP in inhibiting responses to transmural stimulation (Weston, 1973a and 1973b). As has already been discussed, dipyridamole does not reliably potentiate adenosine responses in isolated tissue and the lack of a specific competitive adenosine antagonist precludes the possibility of obtaining definitive evidence of a similarity between adenosine or ATP and nerve-mediated responses using such pharmacological tools. Effects of theophylline on responses to transmural stimulation of the gut have not been reported, but 2-2'-pyridylisatogen does not modify responses to transmural stimulation at doses which markedly antagonise responses to exogenous ATP (Spedding et al., 1975).

Apart from studies involving specific antagonists, the purinergic nerve hypothesis awaits the credibility

that might be imparted if ATP were shown to be stored in the large opaque synaptic vesicles at these nerve teminals. It would also be necessary to show that any ATP in these vesicles is not complexed with another transmitter-like substance. Although the effects of ATP closely mimic those of transmural stimulation, it is possible to interpret the strongest evidence for purinergic nerves as merely indicating that ATP is released from nerve terminals in conjunction with the true transmitter and that ATP can release the transmitter by a tyramine-like action. It must be pointed out, however, that any other possible candidate for the transmitter in this system should be detected in tissue perfusates. None have been reported and thus the hypothesis of purinergic nerves is somewhat more plausible than that of an indirect action of ATP.

1.3. Postulated mechanisms of action of adenosine and nucleotides.

Since adenosine and its nucleotides have been recognised as pharmacologically active substances for almost half a century, it is remarkable that no direct attempt has been undertaken to date to elucidate their mechanism of action on any tissue. Nevertheless, the

following lines of speculation have been initiated by diverse observations enccuntered in the course of other experimentation:

1) Drury & Szent-Gyorgyi (1929) suggested that, since the active compounds known to them were readily deaminated, biological activity might be associated with the alkalinity of the ammonia produced in this reaction.

2) It was suggested by Feldberg & Hebb (1948) and Falck (1956) that adenine nucleotides may relax smooth muscle by chelating or precipitating calcium from the medium, without acting directly on the tissue. On the other hand, Daniel & Irwin (1965) found their results consistent with the hypothesis that ATP and ADP contract uterine muscle by complexing membrane-bound magnesium and thus allowing increased calcium binding and calcium entry into cells. Neither of these proposed mechanisms of action accounts for the fact that in most tissues adenosine, which cannot complex divalent cations, induces qualitatively and sometimes quantitatively similar responses to the nucleotides.

3) As discussed in Section 1.1.2, on the basis of antagonism between adenosine and caffeine, de Gubareff & Sleator (1965) proposed that adenosine prevents the normal action of calcium in excitation-contraction coupling in the myocardium. Axelsson & Holmberg (1969) observed that adenosine and ATP do not modify the electrical activity of

guinea-pig taenia coli previously maintained in calcium-free solution as opposed to normal Krebs and further found that adenine compounds (including adenine which was most potent) relax potassium contractures. Axelsson & Holmberg suggested that adenine, adenosine and nucleotides may relax potassium contractures by interfering with calcium influx into smooth muscle cells. Thus adenosine and nucleotides have so far been discussed as influencing calcium movements only by their action on 'cardiac' receptors and on an unusual system in gut which is responsive to adenine.

4) Tomita & Watanabe (1973) concluded that the hyperpolarisation induced by high concentrations of ATP in guinea-pig taenia coli may be the result of a specific increase in potassium conductance. Since, at lower doses, ATP relaxes the muscle by suppressing spontaneous spike activity without causing hyperpolarisation, this change in potassium conductance is probably not the primary mode of action for ATP and adenosine.

5) In contrast to the suggestion of de Gubareff & Sleator, Schaumann et al. (1970) interpreted antagonism between adenosine and methylxanthines on the heart and coronary circulation as indicating that the drugs may have divergent effects on myocardial cyclic AMP levels. This suggestion followed the publications by Davies (1968) and Stock & Westermann (1969) concerning inhibition of

adenylate cyclase by adenosine and phenylisopropyl-adenosine. Inhibition of adenylate cyclase was similarly proposed by Iso (1973) as the mechanism whereby adenosine potentiates alpha-adrenergic contractile responses in vas deferens, since the potentiation was antagonised by theophylline.

6) The suggestion that adenosine might enhance cyclic AMP accumulation in the myocardium was first made by Kraupp (1969). Later he and his coworkers concluded that a large portion of the coronary vasodilatory response to adenosine was due to propagated metabolic acidosis secondary to adenosine-induced metabolic changes in the myocardium (Raberger et al., 1970 and 1971). These metabolic changes (increased glucose uptake, glycolysis and lipolysis) were similar to those reported to accompany infusion of epinephrine into skeletal muscle vasculature (Lundholm, 1957). Since these metabolic changes are also recognised as being mediated by cyclic AMP in some tissues, Raberger and coworkers proposed that the primary action of adenosine in dilating coronary arterioles is to stimulate myocardial cyclic AMP production. The same group of workers later reported similar effects in dog hind limb following infusion of adenosine into the femoral artery (Raberger et al., 1973a). It is also of interest that Bueding et al. (1967), on the basis of the similarity in responses of guinea-pig taenia coli to epinephrine and to

ATP, suggested a role for extracellular ATP in elevating cyclic AMP levels in intestinal muscle.

1.4. Effects of adenosine on cyclic AMP levels of intact cells or tissue slices.

Although there is no direct evidence that adenosine elevates cyclic AMP levels in the heart, it is well established that adenosine and its nucleotides, including 2'-AMP and 3'-AMP, enhance cyclic AMP accumulation in slices of brain tissue. Adenosine is more potent than the nucleotides but adenine, inosine and other purine nucleosides are inactive (Sattin & Rall, 1970). The effect of adenosine is very pronounced, inducing 20-30 fold increases in tissue cyclic AMP, and is antagonised by methylxanthines. Theophylline is the most effective antagonist but its antagonism can be overcome using higher concentrations of adenosine. Shimizu & Daly (1970), using ^{3}H-adenosine and slices pulse-labelled with ^{14}C-adenine, determined that adenosine elevates cyclic AMP to a small extent by serving as a precursor, but mainly stimulates the accumulation of newly synthesised cyclic AMP. In the same year, Shimizu et al. (1970) found that enhanced formation of cyclic AMP in the presence of depolarising agents is accompanied by, and parallels, release of

adenosine into the incubation medium. It is now accepted that many of the effects of electrical stimulation and of depolarising agents on brain slices, including the ability of the latter to potentiate cyclic AMP accumulation in response to biogenic amines, are mediated by adenosine.

Mutual potentiation between adenosine and biogenic amines implies that these substances act at different sites within the tissue. By investigation of the effects both of inhibitors of adenosine transport and of analogues of adenosine itself, it is currently believed that adenosine acts at a specific extracellular receptor in that tissue, very probably associated with adenylate cyclase (Huang et al., 1972 and Huang & Daly, 1974).

Evidence of extracellular receptor functions with properties very similar to those in brain has been found in cultured cell lines which respond to adenosine with enhanced cyclic AMP accumulation. Schultz & Hamprecht (1973) found that adenosine minimally elevated cyclic AMP levels in neuroblastoma cells and only in the presence of papaverine or methylisobutylxanthine. This effect was not antagonised by theophylline. Blume et al. (1973) found, however, that in the presence of a potent phosphodiesterase inhibitor (Ro 20-1724) adenosine dose-dependently elevated cyclic AMP levels up to 50-fold in

mouse neuroblastoma cells and that the effect was antagonised dose-dependently by theophylline. The dose of adenosine for half-maximal accumulation of cyclic AMP was 2uM, one-tenth of that required in astrocytoma (glial) cells. Clark et al. (1974) also found the effects of adenosine in astrocytoma cells to be very similar to those in brain. The latter workers concluded that antagonism by theophylline was competitive at an extracellular receptor but found no synergism between adenosine and catecholamines. Peck et al. (1974) found synergism between adenosine and either sodium fluoride, epinephrine or parathyroid hormone on cyclic AMP in isolated bone cells; the adenosine effect was antagonised by theophylline.

Adenosine also elevates cyclic AMP in slices of rat lung but not of guinea-pig lung (Palmer, 1971) and in thrombocytes (Mills & Smith, 1971). Methylxanthines antagonised the response in thrombocytes.

In contrast, adenosine has been found to inhibit the accumulation of cyclic AMP induced by epinephrine in intact fat cells (Fain, 1973).

1.5. Effects of adenosine on enzymes related to cyclic AMP.

1.5.1. Adenylate cyclase.

Adenosine inhibits adenylate cyclase isolated from every tissue so far investigated. The only contradictory report indicates that, over a small dose range, adenosine slightly but significantly stimulated cyclase from thrombocytes (Haslam & Lynham, 1972). At concentrations above 0.1mM inhibition ensued. The activation was antagonised by theophylline but not by papaverine and was labile to sonication and repeated freezing and thawing of the enzyme.

Since elucidation of the effect of adenosine on adenylate cyclases comprises a considerable portion of the present work and since most of the literature reports on this subject have appeared during its progress and are relevant to the results of this study, consideration of this topic is dealt with in the discussion and conclusions of this thesis (Sections 3.2.3 and 3.4).

1.5.2. Phosphodiesterase.

Adenosine at millimolar concentrations is a moderately potent inhibitor of phosphodiesterase (Gulyassy, 1971 and Huang & Kemp, 1971). Sattin & Rall (1970) found phosphodiesterase activity in two subcellular fractions of cerebral cortex to be inhibited considerably less by adenosine than by theophylline. No phosphodiesterase has currently been found to be inhibited by adenosine to an extent sufficient to account for the rapid and large increases in cyclic AMP observed in brain slices.

1.5.3. Protein kinase.

Adenosine inhibits cyclic AMP-dependent protein kinase from bovine tissues at concentrations above 10uM (Miyamoto et al., 1969 and Kuo et al., 1970). Iwai et al. (1972) reported that inhibition of rat liver protein kinase was competitive with respect to ATP and was exerted equally on activated and inactivated enzyme.

1.6. The postulated role of cyclic AMP in smooth muscle relaxation.

Following the initial observation by Bueding et al. (1966) that epinephrine elevates cyclic AMP levels in guinea-pig taenia coli at doses inducing relaxation of the muscle, considerable supportive evidence has been obtained to indicate that relaxation of smooth muscle induced by beta-adrenergic agents is preceeded by dose-dependent increases in cyclic AMP. In particular Andersson (1972), using rabbit colon, reported that responses to isoproterenol are accompanied, not only by elevated cyclic AMP levels, but also by an increase in phosphorylase a activity and a reduction in tissue ATP and creatine phosphate levels. Both relaxation and the metabolic effects of isoproterenol were antagonised by beta-blocking drugs. In calcium-depleted muscle, isoproterenol continued to elevate cyclic AMP and phosphorylase a activity but ATP content was no longer diminished and the muscle did not relax. Since accumulation of calcium by a microsomal fraction from rabbit colon in the presence of ATP was found to be stimulated by isoproterenol and cyclic AMP (Andersson & Nilsson, 1972), Andersson has postulated that cyclic AMP promotes the sequestration of cytoplasmic calcium from the vicinity of the contractile apparatus by an ATP-requiring mechanism such as this. It is presumed

that the mode of action of cyclic AMP is through enhanced phosphorylation of membrane protein by a cyclic AMP-dependent protein kinase.

Andersson reported similar metabolic responses in vascular smooth muscle to isoproterenol (1973a) and in vascular and intestinal muscle to phosphodiesterase inhibitors (1973b). Cyclic AMP had already been suggested as the mediator of relaxant responses to phosphodiesterase inhibitors in vascular smooth muscle by Kukovetz & Poech (1970) and Lugnier et al. (1972). Isoproterenol and phosphodiesterase inhibitors act synergistically in these tissues and dibutyryl cyclic AMP mimics not only the relaxant response due to these drugs, but also the accelerated calcium efflux in guinea-pig taenia caeci (Tomiyama et al., 1973) and the hyperpolarisation in pulmonary artery (Somlyo et al., 1970 and 1972) which accompanies the responses. Cyclic AMP-mediated hyperpolarisation was suggested by Somlyo et al. (1972) to be due to stimulation of an electrogenic cation pump.

Work by Andersson (1973a, 1973b and 1973c) has, however, suggested that the relationship between cyclic AMP and smooth muscle tone is not a simple one, but that total cyclic AMP content may reflect variable levels in discrete subcellular compartments. This concept arose

from the finding that agents which induce contraction, although initially decreasing total cyclic AMP in vascular or intestinal muscle (Andersson 1973a and 1973c), subsequently elevated cyclic AMP in a calcium-dependent manner while the muscle remained contracted. Similarly, phosphodiesterase inhibitors, although elevating total cyclic AMP, had variable effects on phosphorylase a activity (Andersson, 1973b), a finding which would be explicable if the phosphodiesterase inhibitors and isoproterenol differentially influenced cyclic AMP in separate compartments including that associated with relaxation. Comparison of cyclic AMP levels in response to various drugs in rat uterus (Polacek & Daniel, 1971 and Polacek et al., 1971) and in rabbit pulmonary artery (Daniel & Crankshaw, 1974) has further indicated that no direct correlation exists between total cyclic AMP content and smooth muscle tone. Nasheim et al. (1975) suggested that cyclic AMP may be only partly responsible for isoproterenol-induced inhibition of rabbit uterine motility.

Despite the complexities which become apparent upon comparing relaxant responses to different drugs with the quantitative increase in total cyclic AMP which they induce, the hypothesis that cyclic AMP mediates responses to beta-adrenergic agonists and at least partially

mediates those to phosphodiesterase inhibitors has gained considerable support (Baer, 1974). While the involvement of other mechanisms in the regulation of smooth muscle contractility is indisputable, modification of cyclic AMP remains a possible basis for the action of drugs on smooth muscle tone, along with more direct effects on calcium or other ion fluxes and probably also with effects on tissue cyclic GMP levels.

1.6. Objectives of this research.

With the intention of providing an insight into the mechanism whereby adenosine relaxes smooth muscle, the central hypothesis was formulated that adenosine acts by elevating cyclic AMP levels in smooth muscle, as it is known to do in brain and other tissues, and that its basic mechanism of action is thus similar to that proposed for isoproterenol. The longitudinal muscle of the rabbit intestine was chosen as the test tissue because it may be separated readily from most extraneous material and because responses to adenosine may be observed in the absence of other drugs or stimulatory agents.

More specifically, the study was intended to:

1) determine whether adenosine and its nucleotides act at

the same receptor site, implying an extracellular site of action,

2) characterise the binding requirements at the site of action in the hope of discovering the properties necessary for competitive antagonism,

3) determine whether adenosine elevates smooth muscle cyclic AMP levels and if so, whether this effect may be causally related to the relaxant response,

4) determine whether a direct effect of adenosine on adenylate cyclase might account for smooth muscle relaxation.

It was hoped that, whatever the results of this study on the rabbit intestinal muscle might be, the findings would be of general validity with respect to other smooth muscles. The ultimate aim was to further our understanding of the proposed physiological roles for adenosine and adenine nucleotides, such as in the control of coronary flow and in the purinergic nervous system.

CHAPTER 2

MATERIALS AND METHODS

2.1. Longitudinal muscle of the rabbit intestine.

2.1.1. Preparation of the tissue.

Male New Zealand White rabbits were killed by injection of 10ml of air into an ear vein and the abdominal cavity was opened. Where 4-12 pieces of tissue were required, as in most experiments, a 20cm length of small intestine was removed starting about 5cm to the oral side of the ligament of Treitz. If more tissue samples were required or for preparation of adenylate cyclase from longitudinal muscle, further 20cm lengths of gut were removed from both oral and anal sides of the ligament. Each 20cm segment was immersed in and gently flushed out with freshly prepared modified Tyrode solution, pH 7.3-7.4, of the following composition: 133.2mM sodium chloride, 4.7mM potassium chloride, 1.9mM calcium chloride, 0.78mM magnesium chloride, 1.17mM sodium dihydrogen phosphate, 18.57mM sodium bicarbonate and 11.11mM dextrose, aerated with 95% oxygen + 5% carbon dioxide (Lui et al., 1969).

Segments were mounted individually on a glass rod of 8mm diameter and the mesentery, along with its underlying layer of longitudinal muscle, was gently removed using fine curved forceps. The edges of a superficial layer of tissue could then be seen on either side of the area from which the mesentery had been separated. Using one limb of a pair of fine notched forceps, one of these edges was gently loosened from the undersurface and the superficial layer was then rolled back and removed using a paper tissue moistened in Tyrode solution. Throughout this procedure the entire segment was periodically moistened with Tyrode solution to prevent drying out. The superficial layer thus prepared was cut into 8-12 strips approximately 8-10mm in length and extending about 2cm in breadth. The end regions were discarded. Each strip was used as one sample of longitudinal muscle. On electronmicroscopic examination, the tissue was found to be comprised of serosa, longitudinal muscle, Auerbach's plexus and one or two cell layers of circular muscle (Figure 1).

Strips to be used immediately were suspended in Tyrode solution at 37°C while those required for subsequent study were stored in Tyrode at 4°C for up to 3 hours or for longer periods as required for determination of the effect of cold storage.

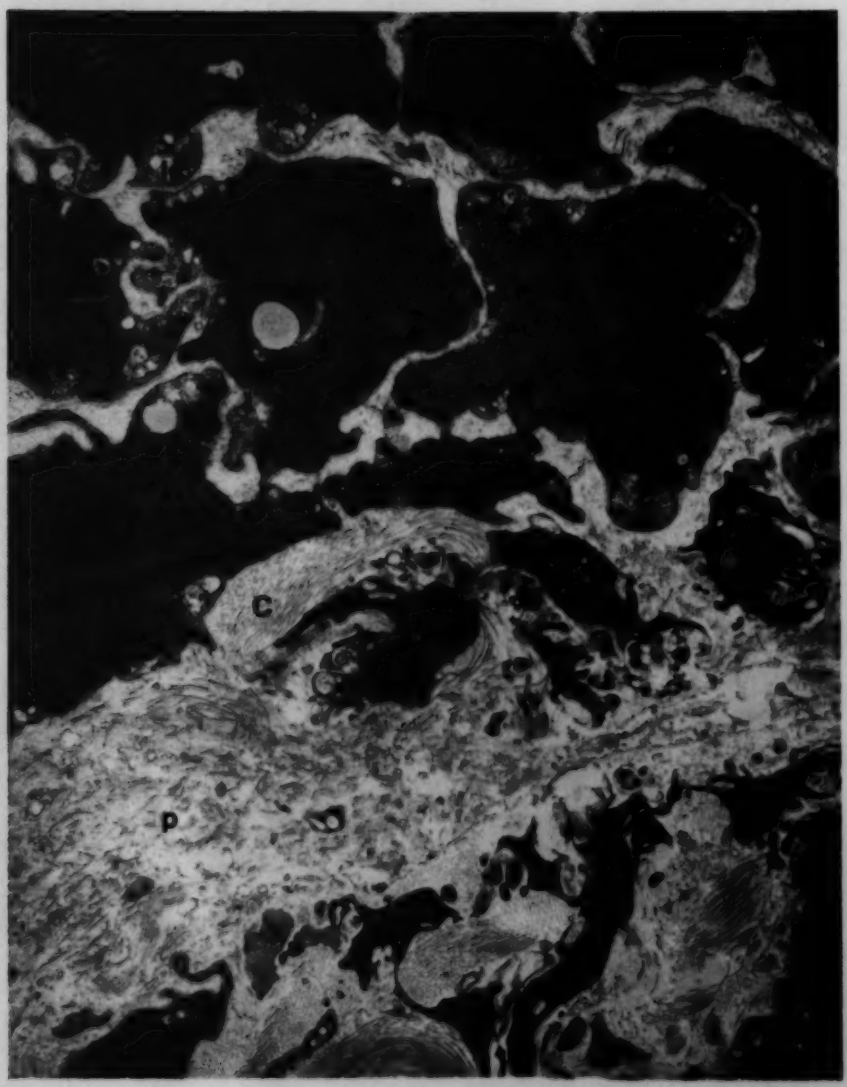


Figure 1: Electron micrograph of the experimental tissue.
Transverse section, magnification ×18,000. sm: longitudinal smooth muscle cell; ic: intermediate contact; p: Auerbach's plexus; n: nerve; C: collagen.

2.1.2. Measurement of relaxant response.

The apparatus used for recording mechanical activity of the muscle is illustrated in Figure 2. Four pairs of lubricated vertical rods were fixed to a solid wooden structural frame immediately below four Grass force-displacement transducers, type FT03C, whose position could be altered using a rack-work boss head (C.P. Palmer, London, Ltd.). Each pair of rods supported a plexiglass block into which a jacketed organ bath of 17ml capacity was fitted. The organ bath was secured at the top of the rods, in the 'normal' position, by means of a metal pin attached to a lever. Small springs were interposed between the plastic block and the upper rod support to provide a spring-loaded dropping action of the organ bath when the lever was turned. A metal tissue support, which also served as aerator, was fixed to the frame and extended to the bottom of the organ bath in the 'normal' position. The outer jackets of the organ baths were connected in parallel to a Thermomix II thermostatic pump (Braun Melsungen) circulating water from a reservoir. Fresh Tyrode solution was warmed by the reservoir and added manually to the organ baths. Drainage from the organ bath was effected through an outlet secured with a spring clip.

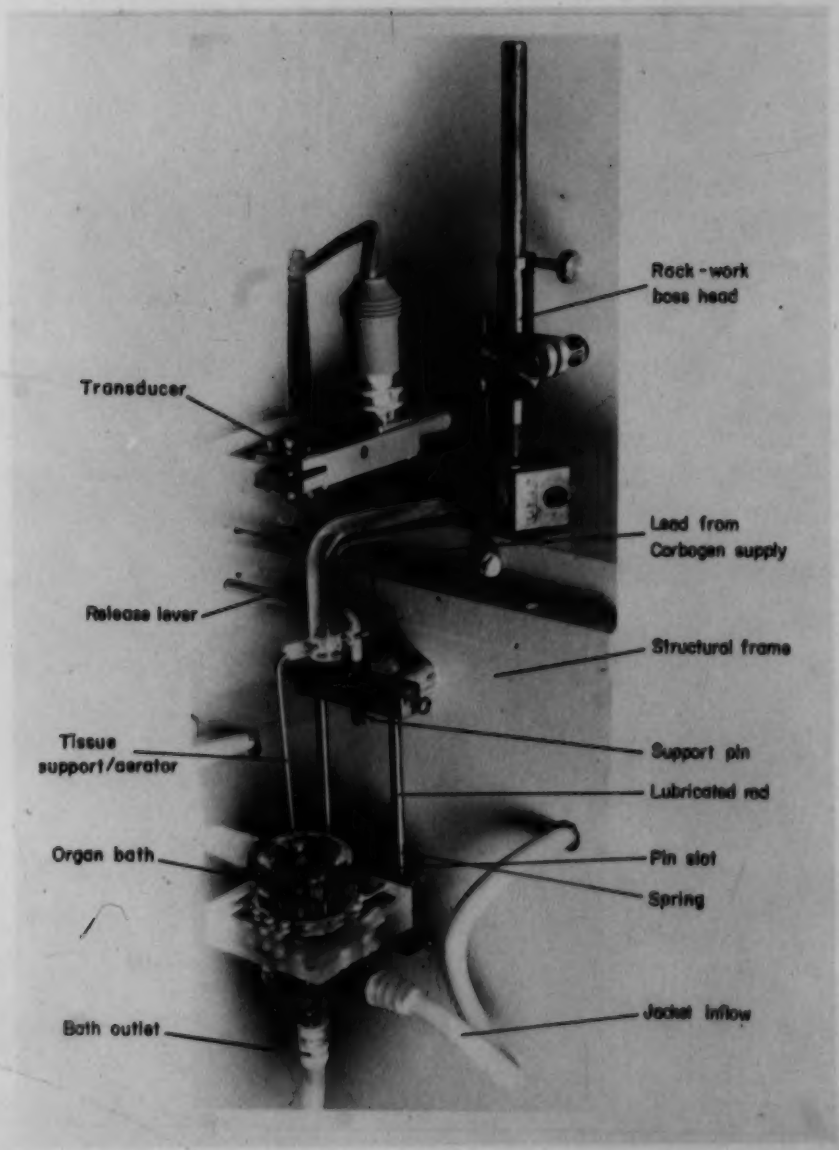


Figure 2: Apparatus for measurement of relaxant responses and rapid tissue sampling.
The apparatus is illustrated in the position for tissue sampling.

Drug solutions were injected by syringe into the bottom of the organ bath in volumes of 0.1 to 0.5ml, to produce a total bath volume of 17ml. The bath contents were mixed by the carbogen flow. Stock drug solutions were dissolved in calcium-free Tyrode and diluted before use with aerated normal Tyrode solution. Solutions of sympathomimetic drugs also contained 10ug/ml ascorbic acid. Stock solutions of adenine nucleotides were neutralised with NaOH.

Each strip of longitudinal muscle was suspended with thread, in Tyrode solution at 37°C, between the tissue support and a force-displacement transducer which was linked via a Beckman strain gauge coupler type 9853 to a 4-channel Beckman type RB Dynograph. Strips were allowed to equilibrate under minimal tension (<0.1g) for at least one hour. Since this muscle is spontaneously active and the amplitude of spontaneous contractions depends on the imposed tension, the tension in each strip was then gradually increased by elevating the position of the transducer until the optimal amplitude of spontaneous contractions corresponding to a pull of approximately 1g (2.5cm excursion of the pen) was achieved. Applied tension was about 0.3g.

Inhibitory responses to smooth muscle relaxant drugs

consisted of a depression of the amplitude of spontaneous contractions, frequently, but not invariably, accompanied by a decrease in the basal tension of the strip. The latter was also dependent on the imposed tension. Since optimal spontaneous activity was achieved by different strips at applied tensions of from 0.2 to 0.4g and inhibition of basal tension in response to drugs consequently varied between strips, the smooth muscle relaxant response was defined for the purpose of this study as inhibition of spontaneous activity. The magnitude of the response was expressed as inhibition of the amplitude of spontaneous contractions, as a percentage of the control amplitude immediately preceeding drug addition (Figure 3).

2.1.3. Measurement of tissue cyclic AMP levels.

2.1.3.1. Tissue sampling.

The apparatus illustrated in Figure 2 was designed to allow rapid freezing of tissue samples with minimal derangement of the tissue in the organ bath. Muscle strips whose responses were being recorded were frozen for estimation of their cyclic AMP content by releasing the lever to drop the organ bath and clamping the tissue

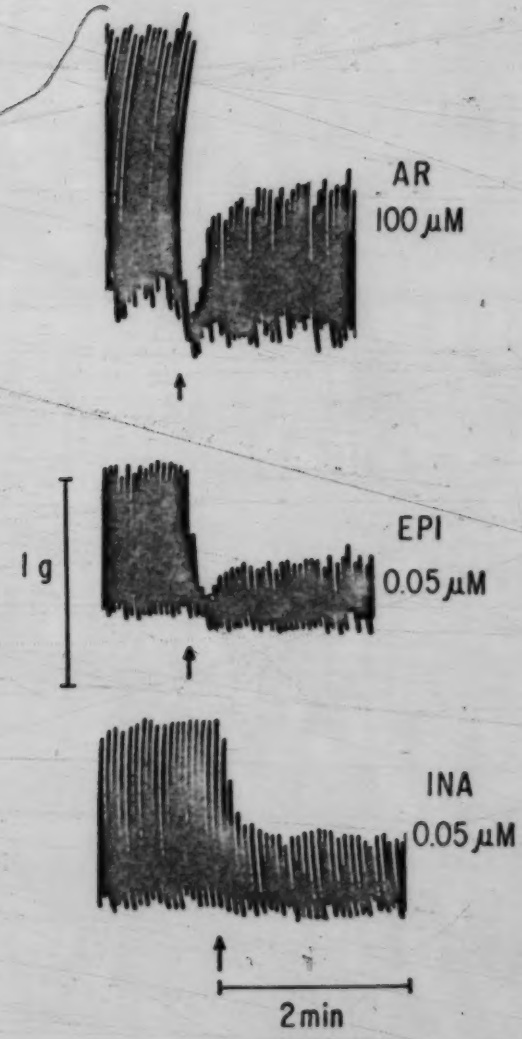


Figure 3: Responses to adenosine, epinephrine and isoproterenol. Responses were evaluated as inhibition of the amplitude of spontaneous contractions and expressed as a percentage of the control amplitude immediately preceeding drug addition.

between heavy metal tongs precooled to the temperature of liquid nitrogen. The tongs consisted of two brass bars, approximately 30x10x3mm, soldered to a pair of forceps. The total time required for this sampling procedure was less than 2 seconds, inclusive of time for freezing. At the time these studies were commenced, rapid freeze-clamping methods had not been applied to smooth muscle. This procedure has been demonstrated in many other systems to avoid the rapid changes in cyclic AMP during sampling which are due to slow inactivation of adenylate cyclase and phosphodiesterase.

In experiments in which multiple samples were exposed to identical treatments, the strips were tied together with thread and were not attached to the transducer or the tissue support. Freezing was accomplished in this case by rapid removal from the organ bath and immersion in liquid nitrogen.

2.1.3.2. Homogenisation.

Tissues remained frozen until after homogenisation (see flow sheet, Figure 4). Individual strips were enclosed in a Teflon capsule containing 0.3ml 5% TCA and a tungsten carbide ball, all at the temperature of liquid

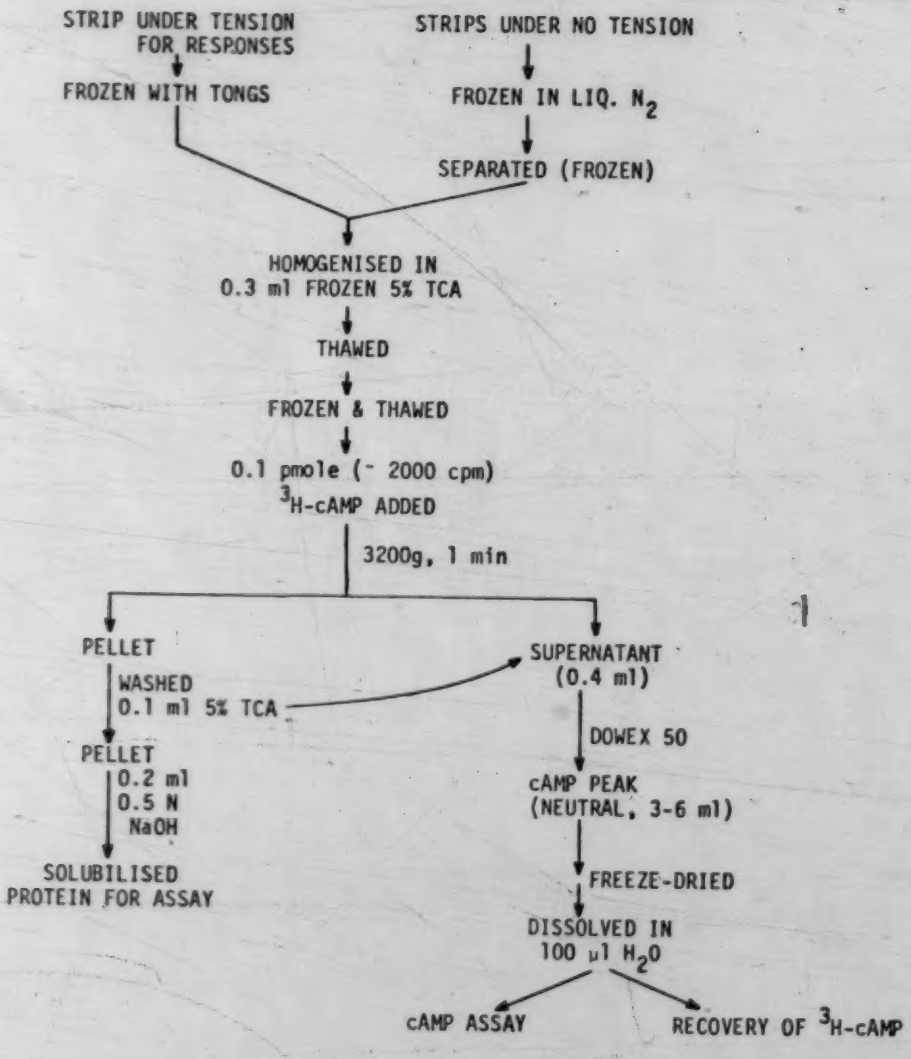


Figure 4: Flow diagram of preparatory procedure for measurement of tissue cyclic AMP levels.

nitrogen. The capsule was fitted to a Mikro-Dismembrator (Braun Melsungen) and vibrated at 60 Hz with an amplitude of 4-5mm for 30 seconds. The homogenate powder was collected in small centrifuge vials (Eppendorf) and allowed to thaw. After subsequent freezing and thawing, 0.1pmole of tracer ^{3}H-cyclic AMP (approximately 2000 cpm) was added to the homogenate and the TCA-soluble supernatant was removed following centrifugation for 1 min in an Eppendorf 3200 bench centrifuge. The protein pellet was washed with 0.1ml 5% TCA and the supernatants pooled.

2.1.3.3. Purification.

The TCA extract was applied to a 0.5x7cm column of Dowex AG50W-X4, hydrogen form, (100-200 mesh) (Bio-Rad) previously acid-washed and equilibrated with distilled water and the sample was eluted with successive 0.5ml volumes of distilled water. The TCA eluted rapidly while cyclic AMP was retained and its peak, collected in a 3-6ml volume, depending on the resin batch, was neutral (Figure 5). The position of the cyclic AMP peak was unaffected by the presence of either tissue extract or 5% TCA in the applied sample.

When 50ul ATP or 100ul 5'-AMP at approximately 10mM

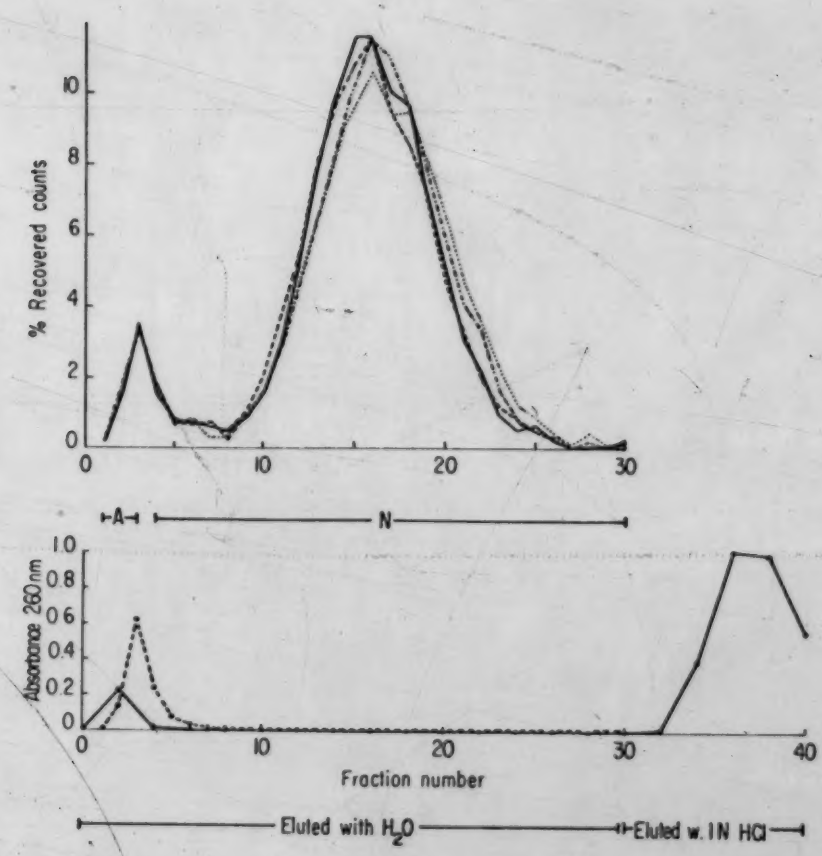


Figure 5: Elution profile from Dowex of ^{3}H-cyclic AMP, ATP and 5'-AMP. 0.5ml fractions. Upper curve: recovery of radioactivity from ^{3}H-cyclic AMP in samples containing extract from tissues of the following protein content: (····) 8.4mg; (·-·-) 15.6mg; (----) 22.1mg; (——) control containing no tissue extract. Lower curve: absorbance at 260nm of eluate fractions after application of: (····) 50ul ATP; (——) 100ul 5'-AMP at 10mM and diluted to 0.4ml with 5% TCA.

were included in the TCA sample and the absorbance of a 1:10 dilution of eluate fractions was read at 260nm on a Gilford spectrophotometer it was found that complete separation of these nucleotides from cyclic AMP was effected (Figure 5). Since 5'-AMP was not completely eluted by 5ml of 1N HCl and since adenosine is retained even more effectively on cation exchange resins the columns were used only once.

The position of the cyclic AMP peak was monitored occasionally by counting ^{3}H-cyclic AMP in successive 1ml eluate fractions dissolved in 10ml Aquasol (New England Nuclear) or Bray's solution (60g/l naphthalene, 20ml/l ethylene glycol, 100ml/l methanol, 4g/l 2,5-diphenyl-oxazole (PPO), 0.2g/l 1,4-bis-2-(4-methyl,5-phenyl-oxazole)-benzene (dimethyl POPOP, Packard) in 1,4-dioxane) and in subsequent experiments the cyclic AMP peak was collected as indicated by the most recent test run.

The cyclic AMP peak from the Dowex columns was freeze-dried and redissolved in 100ul water for use in the cyclic AMP assay and for determination of recovery of tracer ^{3}H-cyclic AMP.

2.1.3.4. Assay of cyclic AMP

Cyclic AMP was assayed by a protein binding method (Gilman, 1970) utilising a cyclic AMP-binding protein prepared from beef heart by G.J. Lauzon in this laboratory, according to the method of Miyamoto et al. (1969). 'Inhibitor protein', which is recommended by Gilman as a means of increasing the sensitivity of the assay, was prepared from beef skeletal muscle by L. Simonson using the procedure of Appleman et al. (1966). The assay medium contained in a total volume of 100ul: 50mM sodium acetate, pH 4; 20ug bovine serum albumin (Sigma); 19ug inhibitor protein; 1.5ug binding protein; 0.2pmole ^{3}H-cyclic AMP (4000 cpm) and unlabelled cyclic AMP in duplicated standard or unknown (10 or 20ul of tissue sample) quantities. Blanks, containing no binding protein, and standards, containing 0.5 to 20pmoles added cold cyclic AMP, were included in each assay and unknown quantities of cyclic AMP from tissue samples were read from the straight line part of the standard curve obtained in that assay. Standard curves (Figure 6) were highly reproducible.

The equilibration reaction was initiated by addition of binding protein and assay tubes were allowed to equilibrate for at least 90 minutes at 4°C. Each assay

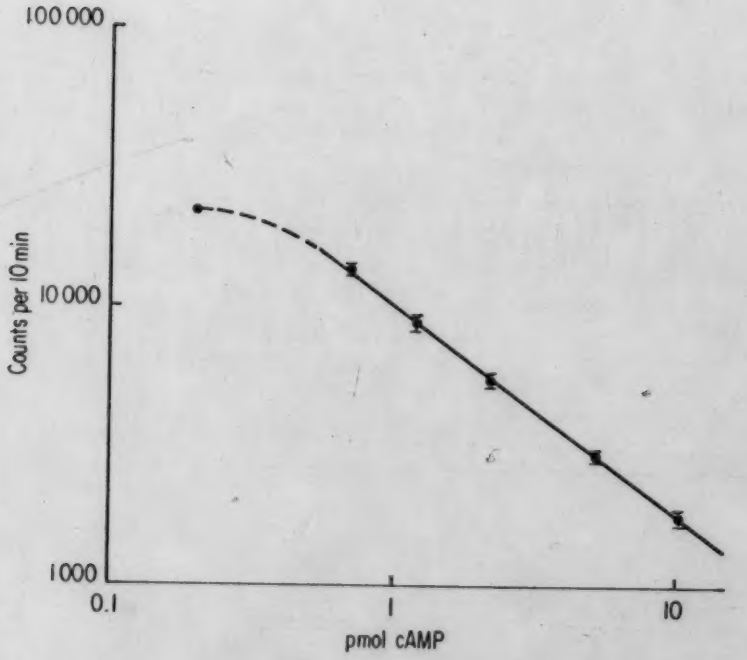


Figure 6: Standard curve from cyclic AMP assay.

medium was then filtered under vacuum at 4°C through a 25mm diameter membrane filter of 0.45 micron pore size (Matheson-Higgins, Woburn, Mass.) by the following procedure. The tube contents were diluted with 1ml of 20mM sodium phosphate buffer, pH 6, and immediately passed through the filter. The tube was washed twice in a similar manner with 1ml aliquots of buffer and the filter was subsequently washed twice with 4ml of the same buffer. The filter discs were placed in scintillation vials and dissolved in 1.8ml ethylene glycol monomethyl ether. The vials were filled with 5ml toluene fluor (5g/l PPO and 0.25g/l dimethyl POPOP in toluene), shaken until mixed and counted for tritium in a Beckman LS330 or Picker Nuclear Liquimat scintillation counter. The efficiency of counting was approximately 30%, as determined using quenched external standards, and was constant within each experiment. Calibration curves as in Figure 6 were therefore plotted on double logarithmic paper as counts per 10min versus total cyclic AMP.

To ensure that no component of the tissue sample was interfering with the assay of cyclic AMP, periodic assay checks were performed. In this procedure aliquots of tissue samples were preincubated with phosphodiesterase to destroy their cyclic AMP content. As recommended by Ebadi (1972), phosphodiesterase was prepared from hog cerebral

cortex by the method described by Nair (1966) for dog heart. The method is described in Figures 7 and 8. The final column chromatographic step in the published procedure was omitted. Although Ebadi describes this method as applicable to hog brain despite the differences in species and tissue (personal communication), in our hands this method was unsatisfactory in that the yield of fraction V represented only 0.13% of fraction I (Table 1). Most activity was lost during the first ammonium sulphate fractionation where 39.1% yield was found in the first cut and 26.5% in the supernatant after equilibration with 62% ammonium sulphate. In spite of this low yield, the amount of enzyme obtained by this procedure was sufficient for all necessary analytical experiments.

Fractions were assayed by recording the change in optical density at 265nm on a Gilford 2400 spectrophotometer of an assay mixture containing 50mM Tris-HCl (pH 8), 5mM magnesium chloride, 50mM cyclic AMP, 20ug bacterial alkaline phosphatase (type III from E. coli Sigma), 4ug adenosine deaminase (type I from calf intestinal mucosa, Sigma) and phosphodiesterase in a total volume of 2ml. The assay was performed at room temperature. The difference in absorbancy at 265nm between adenosine and inosine is 7.85×10^3 l/mole.cm at pH 8 (Baer et al., 1968). Using this value, absolute rates

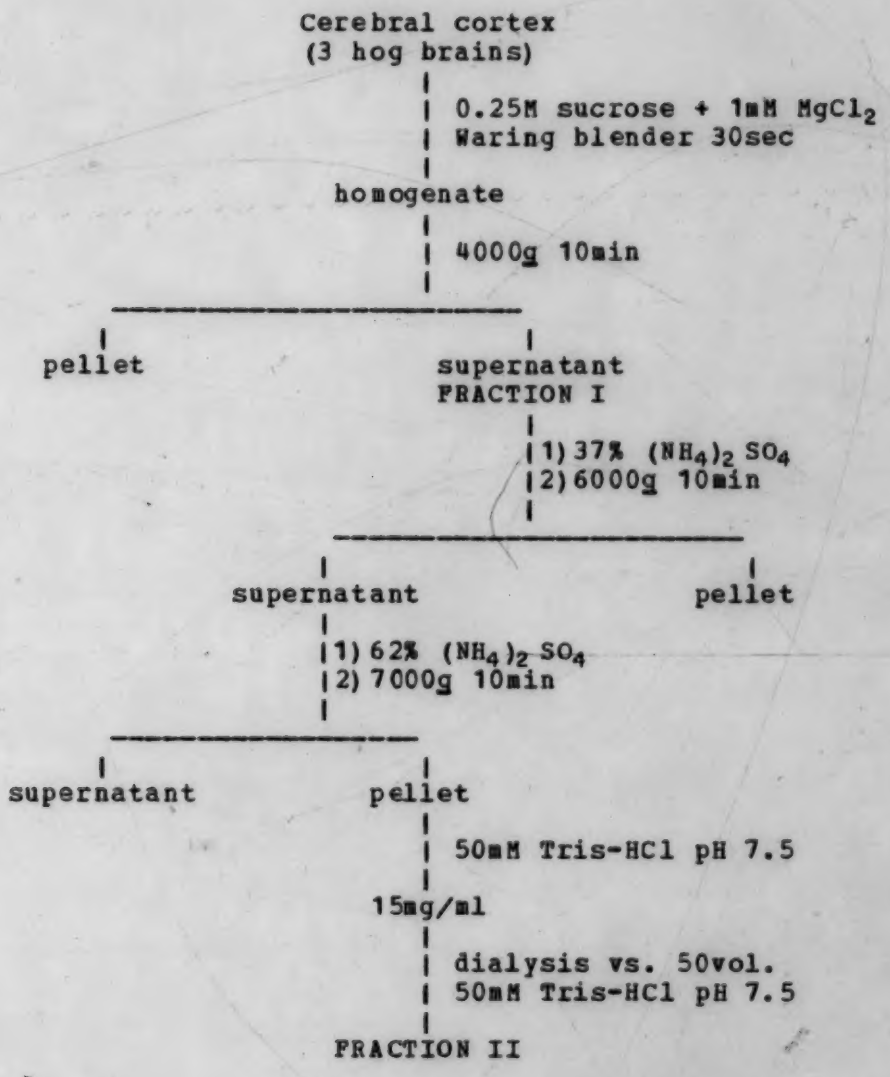


Figure 7: Preparation of phosphodiesterase. Part I.
Cerebral cortex from 3 hogs was homogenised and the 4000g supernatant was subjected to ammonium sulphate fractionation. The pellet obtained between 37 and 62% saturation was redissolved at 15mg/ml and dialysed against the same buffer.

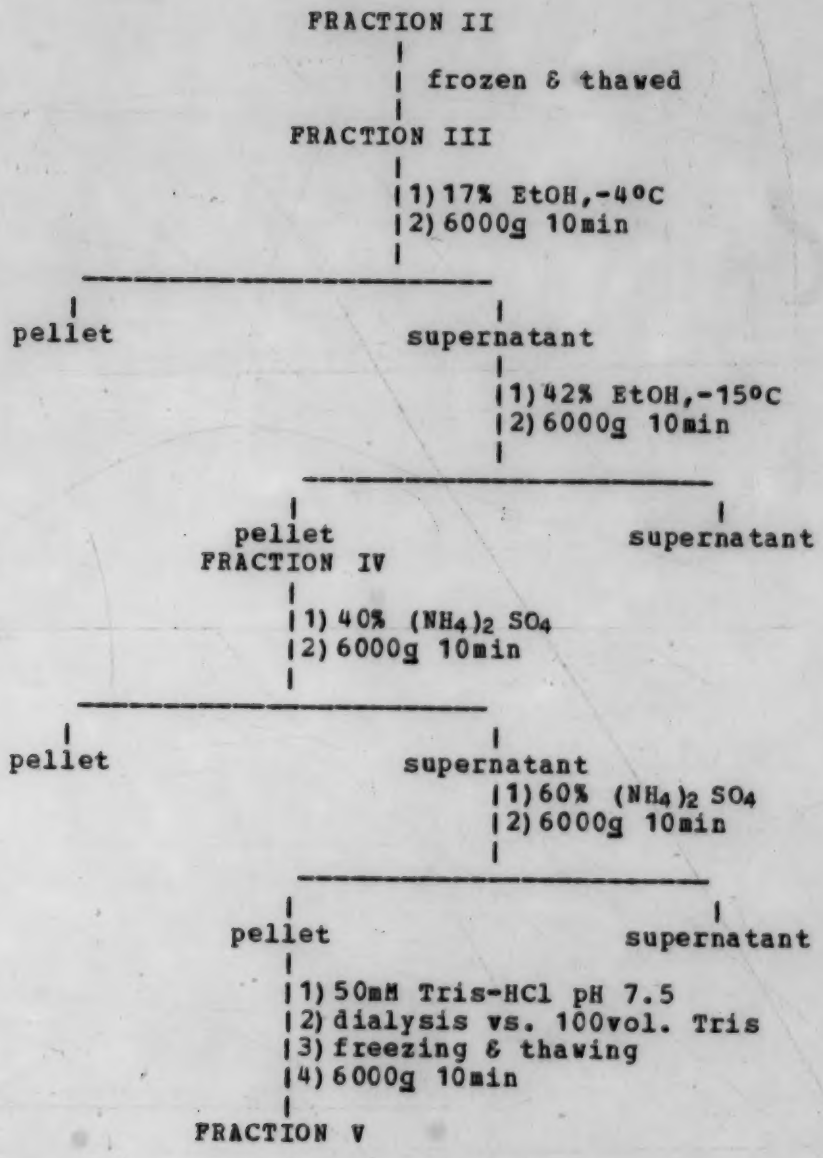


Figure 8: Preparation of phosphodiesterase, Part II. Fraction II was frozen and thawed and subjected to ethanol fractionation. The 17-42% EtOH precipitate was further fractionated between 40 and 60% ammonium sulphate. After dialysis, freezing and thawing and removing precipitated material, fraction V, the phosphodiesterase used to prepare cyclic AMP blanks, was obtained.

Fraction	Protein (mg/ml)	Specific Activity (nmole/mg/min)	% Yield
I	9.0	4.21	100
II	11.9	1.51	8.2
III	7.7	2.18	7.5
IV	14.0	6.75	0.55
V	5.6	6.76	0.13

Table 1: Specific activity and yield of fractions during preparation of phosphodiesterase.
Fractions are as designated in Figures 7 and 8.

were calculated from the initial rate of change of absorbance of the mixture. Since phosphodiesterase activity was rate-limiting under these conditions, the rate of the deamination reaction reflected the rate of cyclic AMP hydrolysis by phosphodiesterase. The specific activity of fraction V was calculated by this method to be 6.76nmole/mg/min. The enzyme was stored in small aliquots at -20°C and diluted 1:10 with 1% bovine serum albumin (fraction V, powder, Sigma) before use.

To determine whether this preparation would adequately hydrolyse cyclic AMP contained in tissue samples, duplicate assay tubes were prepared containing 4mM Tris-HCl (pH 7.5), 0.8mM magnesium chloride, 0.2pmole ^{3}H-cyclic AMP, cold cyclic AMP (2pmole or 10ul tissue extract) and 5ul phosphodiesterase diluted 1:10 (3ug enzyme protein) in a total volume of 25ul. Controls, in which unlabelled cyclic AMP and phosphodiesterase were omitted, were also included. The tubes were sealed to minimise volume loss and incubated at 37°C for 30 minutes and the reaction was stopped by immersion in boiling water for 2 minutes. The incubates were then spotted, with the appropriate carriers, on Whatman No.1 chromatographic paper and the chromatograms were developed in 1M ammonium acetate:95% ethanol 30:75 for 12 hours. Spots corresponding to 5'-AMP, cyclic AMP and adenosine plus

inosine were cut out, eluted with 1ml water and counted in Aquasol for tritium and the percentage of counts in each spot was calculated. Results of duplicates are shown in Table 2. All concentrations of cyclic AMP were hydrolysed over 90% by 3ug enzyme. Since the breakdown products were predominantly nucleosides, the preparation also contained phosphatase. Although Nair states that this contaminant may be removed by elution of fraction V through DEAE, it was decided, owing to the small yield and because the preparation was adequate for the intended purpose, not to pursue this step. It is interesting that the tissue extract apparently inhibited phosphatase, probably due to phosphate esters within the extract competing with 5'-AMP as substrate for the reaction. The blanks show that 94% of the radioactivity in ^{3}H-cyclic AMP was located by this method in the cyclic AMP spot.

For the purpose of checking the validity of the cyclic AMP assay, 10ul aliquots of tissue extract were preincubated in duplicate for 30 minutes at 37°C with 5mM Tris-HCl (pH 7.5), 1mM magnesium chloride and 3ug phosphodiesterase in a total volume of 20ul. The sealed reaction vessels were immersed in boiling water for 2 minutes to stop the reaction. These tubes were then included in the cyclic AMP assay, along with tubes containing 10 and 20ul of tissue extract. No cyclic AMP

	pmoles cyclic AMP			
	Blank	0.2	2.2	0.2+extract
% cyclic AMP breakdown	5.9	94.3	92.7	92.0
	5.9	93.9	93.5	90.2
% AMP	1.0	8.4	15.5	86.4
	0.9	13.4	12.5	85.0
% Nucleosides	4.8	85.8	77.2	5.6
	5.0	80.5	80.9	5.1

Table 2: cyclic AMP breakdown in phosphodiesterase-treated samples.
Breakdown was determined as described in the text and expressed as total percentage breakdown and percentage counts in the AMP and nucleoside spots.

was detected in tissue blanks and total cyclic AMP determined in extracts was independent of the volume of extract used in the assay.

2.1.3.5. Expression of results.

Since tissue samples included variable amounts of Tyrode solution owing to the rapid sampling technique, an estimate of tissue wet weight could not be obtained. TCA-insoluble material was therefore dissolved in 0.2ml of 0.5N sodium hydroxide and protein was determined by the method of Lowry et al. (1951). Cyclic AMP content of tissue samples was expressed as pmoles cyclic AMP per mg acid precipitable tissue protein.

2.1.4. Preparation of adenylate cyclase.

When attempts were made to prepare adenylate cyclase from longitudinal muscle using the general method described in Section 2.2, the protein sedimented from suspension and showed a marked tendency to coagulate, especially upon freezing and thawing. This difficulty was probably due to the relatively large amounts of collagen associated with smooth muscle. To circumvent this

problem, adenylate cyclase was prepared by an adaptation of the method described by Rodbell (1964 & 1967) for preparation of cyclase from adipose tissue. Longitudinal muscle was dissected as described in Section 2.1.1 from the entire length of the small intestine, yielding just over 1g tissue per rabbit. The tissue was maintained throughout dissection in ice-cold Tyrode. After mincing finely with scissors, the tissue was incubated with 5 volumes of collagenase medium (containing 140mM sodium chloride, 9.1mM sodium phosphate, 5mg/ml collagenase (type I from Cl. histolyticum, Sigma), 1mg/ml dextrose and 5mg/ml bovine serum albumin at pH 7.4) at 37°C for 3 hours during which time it was shaken at 140 cycles per minute. The suspension was filtered through nylon netting and the residue was washed with phosphate buffer (140mM sodium chloride 9.1mM sodium phosphate and 5mg/ml lyophilised bovine serum albumin, pH 7.4). The filtrate was centrifuged at 50% maximal speed on an International Clinical Centrifuge for 2 minutes and sedimented cells (0.1 to 0.2ml packed cell volume per gram tissue) were washed twice with 5 volumes of phosphate buffer. At this point in the procedure individual spindle-shaped cells could be observed microscopically under phase contrast. Since the individual cells were fragile to mechanical damage, resuspension was performed gently using a vortex mixer. The final pellet of cells was allowed to stand in

5 volumes of 10mM Tris-HCl pH 7.5 + 1mM magnesium chloride at 4°C for 10 minutes and was subsequently homogenised gently in a glass homogeniser with a Teflon pestle. No intact cells could be seen under phase contrast at this stage. The homogenate was centrifuged at 1000g for 15 minutes and the pellet was washed and resuspended in a small volume of the same buffer. Using this procedure, the protein neither sedimented nor coagulated and fluoride-stimulated activity was over 100pmole cyclic AMP/mg protein/min.

Since the possible contaminants of cyclase preparations which can interfere with the assay of adenylate cyclase activity are ATPase and phosphatase, which reduce the concentration of substrate, and phosphodiesterase, which hydrolyses the cyclic AMP^{32} product of the reaction (see Section 2.2.2 for a description of the adenylate cyclase assay procedure), the following experiment was performed to determine the optimal assay conditions for this enzyme. Four tubes were prepared containing, in a total volume of 200ul:

Tube A: reagents in the concentrations regularly used, including 10mM creatine phosphate, 0.1mg/ml creatine kinase and 0.5mM cyclic AMP

Tube B: as tube A but containing 20mM creatine phosphate and 1.1mg/ml creatine kinase

Tube C: as tube B but also containing 10mM NaF

Tube D: as tube B but containing 1mM cyclic AMP.

All tubes also contained 0.27mg/ml of freshly prepared enzyme protein and were incubated at 37°C. Aliquots of 2-4ul were withdrawn from each tube 0, 10, 20, 30 and 40 minutes after addition of enzyme and were immediately spotted on PEI plates over the carriers appropriate for determining percent conversion of ATP to cyclic AMP (EDTA-stop solution, Section 2.2.2) or percentage ATP breakdown (5mM ATP, ADP, AMP and cyclic AMP). Plates were developed in 0.25M lithium chloride to determine cyclase activity or in 1M LiCl for ATP breakdown. The appropriate spots were cut out and counted for ^{32}P in 15ml toluene fluor. Results are shown in Figure 9. In the absence of fluoride, 90% ATP breakdown occured over 40 minutes, indicating that ATPase and phosphatases were troublesome contaminants of the cyclase preparation. Breakdown could not be reduced by increasing the ATP-regenerating capacity of the medium, but it was halved in the presence of NaF. This effect no doubt contributed to the greatly enhanced conversion of ATP to cyclic AMP in the presence of fluoride. Doubling the concentration of unlabelled cyclic AMP to further reduce any phosphodiesterase interference in the cyclase assay did not appreciably modify either ATP breakdown or conversion of ATP to cyclic AMP. Since the rate of cyclic AMP production was linear over 20 minutes

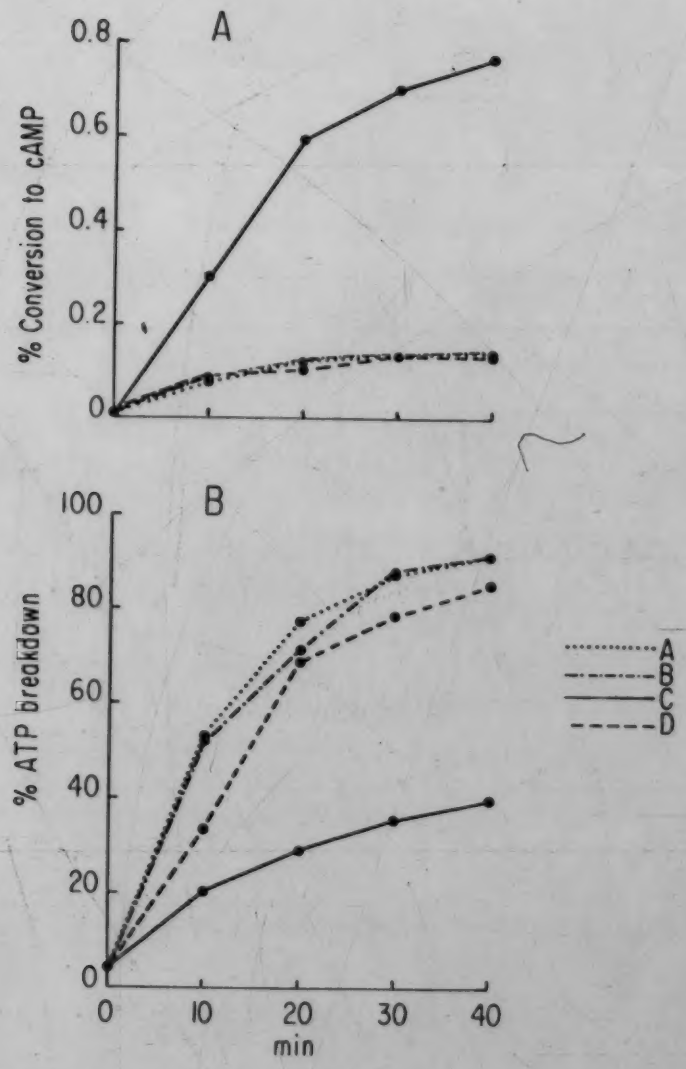


Figure 9: Cyclic AMP formation and ATP breakdown by an adenylate cyclase preparation from longitudinal muscle.
Determinations from single tubes, as described in the text. A: conversion of ATP to cyclic AMP. B: total ATP breakdown.

in the presence of fluoride, cyclase assays could be performed within this time interval.

2.2. Preparation and assay of adenylate cyclase from various tissues.

2.2.1 Preparation of adenylate cyclases.

Adenylate cyclase was prepared in the form of 2000g (10min) washed pellets from whole rat brain, rat cerebellum and cerebral cortex, rat heart, rabbit heart, dog thrombocytes and guinea-pig lung by essentially the same method. Ehrlich ascites cell cyclase was prepared by others in this laboratory according to Baer & Henderson (1972) and ghosts from rat epididymal fat cells according to Rodbell (1967). Tissues were homogenised in 5 volumes of 20mM Tris-HCl, pH 7.5 + 1mM magnesium chloride using a glass homogeniser with a power-driven Teflon pestle. After filtration through glass wool the homogenates were centrifuged at 2000g for 10 minutes and the pellets were washed with 5 volumes of buffer and stored under liquid nitrogen in small aliquots. The preparations were used either freshly prepared or after freezing and thawing once. The extent of conversion of ATP to cyclic AMP and ATP breakdown as a function of time were determined for

each tissue and incubation time and protein concentrations were then chosen to ensure linearity of cyclic AMP production. The protein content of each preparation was determined by the method of Lowry et al. (1951).

2.2.2. Assay of adenylate cyclase activity.

The assay procedure was essentially similar to that described by Baer & Hechter (1969) with improvements according to Baer (1975). Reaction mixtures contained, usually in a 50ul total volume, 25mM sodium N-2-hydroxymethylpiperazine-N'-2-ethanesulphonate (pH 8), 5mM magnesium chloride, 0.5mM sodium cyclic AMP, 0.1mg/ml creatine kinase, 10mM sodium creatine phosphate, 0.1mM ATP-alpha-^{32}P (about 500,000 cpm) enzyme and other additions as required in duplicate or triplicate. For rate studies the ATP concentration was adjusted by addition of known quantities of unlabelled ATP. Unless otherwise indicated, as in reversal of adenosine inhibition by adenosine deaminase, the reaction was initiated by addition of enzyme and terminated by addition of ice-cold EDTA-stop solution (125mM EDTA and 25mM each of ATP, 5'-AMP and cyclic AMP, pH adjusted to 7 with sodium bicarbonate) and immersion of reaction vessels in ice. A 3-4ul aliquot from each reaction vessel was spotted over a 1.5cm-wide strip on washed 10x20cm CEL300

PEI plates (polyethyleneimine-impregnated cellulose thinlayers from Macherey-Nagel/Brinkman). Plates were developed in 0.25M LiCl for the full 10cm and spots corresponding to ATP, 5'-AMP and cyclic AMP were located under ultraviolet light. The spots containing cyclic AMP and ATP plus AMP were placed in scintillation vials and counted in 10ml toluene fluor. The percent conversion of ATP to cyclic AMP was calculated from the ratio of counts in each area and results were expressed as pmoles cyclic AMP formed per mg protein per minute.

2.3. Chemicals.

All chemicals were of reagent grade and dissolved in deionised distilled water. Ammonium sulphate used in the preparation of phosphodiesterase was enzyme grade (Schwarz/Mann). Adenosine and its nucleotides (sodium salts), 2'-deoxyadenosine, tris(hydroxymethyl)aminomethane (Trizma base), aminophylline, D,L-isoproterenol HCl and L-epinephrine bitartrate were purchased from Sigma.
Adenosine 5'-triphosphate, tetrasodium salt, alpha-^{32}P, was purchased at 10Ci/mmole from International Chemical and Nuclear Corp. and diluted approximately 20 fold with 1mM ATP before use.
Adenosine-^{3}H(G)-3',5'-cyclic monophosphate (ammonium salt) in 50% ethanol (24 Ci/mmole) was purchased from New

England Nuclear.

3'-Deoxyadenosine and 2-amino-6(p-nitrobenzylthio)-purine riboside were gifts from Dr. F. Henderson and Dr. A.R.P. Paterson, respectively, of the McEachern Cancer Research Institute, University of Alberta.

Other sources were as follows:

N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), A grade: Calbiochem, USA.

Adenosine analogues:

Pharma Waldhof, GmbH, West Germany.

Creatine phosphate disodium salt and creatine kinase:

Boehringer Mannheim, GmbH, West Germany.

Alpha,beta-methylene adenosine diphosphate (APCP):

Miles Laboratories, USA.

1-Methyl,3-isobutylxanthine (MIX):

Aldrich Chemical Co. Inc., USA.

Papaverine HCl: Eli Lilly & Co., USA.

Dipyridamole (Persantin):

Geigy Pharmaceuticals, Montreal, Canada.

Procaine HCl: Matheson, Coleman & Bell, USA.

Lidocaine (Xylocaine HCl, 4%):

Astra, Ontario, Canada.

CHAPTER 3

RESULTS AND DISCUSSION.

3.1. Relaxant responses.

As indicated in Section 1.7, the objectives of these experiments were:

1) to compare the influence of various experimental conditions on responses to adenosine and its nucleotides, particularly ATP, in order to determine whether they share a common extracellular site of action.

2) to screen a series of available adenosine analogues in order to determine which moieties are necessary for adenosine-like activity and also to seek a competitive antagonist of adenosine responses.

This experimentation was preliminary to the anticipated quantitative evaluation of the effects of drugs on adenosine-induced relaxant responses and concomitant changes in cyclic AMP content of tissues and, unless otherwise indicated, the drug effects reported in this section are results obtained from only two to three animals.

3.1.1. Adenosine and analogues.

3.1.1.1. The adenosine response.

Responses to adenosine in longitudinal muscle strips were biphasic (Figure 3). An initial transient inhibition of the amplitude of spontaneous contractions achieved a maximal value in 10-15s and was followed by a sustained response of lesser magnitude which was usually constant within 1 minute. If a maximal dose was left in contact with the tissue, recovery toward control amplitude of contractions was only about 50% within one hour. There was no change in the frequency of spontaneous contractions. Both initial and sustained responses were dose dependent over a dose range which varied slightly between animals and between strips from one animal but which generally lay between 0.1 and 100uM (Figure 10). The initial response appeared at slightly lower doses than the sustained response and the maximal sustained response attainable also varied between tissues. Following washout after 2 minutes of exposure to adenosine, spontaneous activity rapidly returned to the control level and frequently exceeded it for a few minutes. This 'rebound overshoot' was not invariably present. Successive doses were administered when control levels were regained, at intervals of not less than 10 minutes. No tachyphylaxis

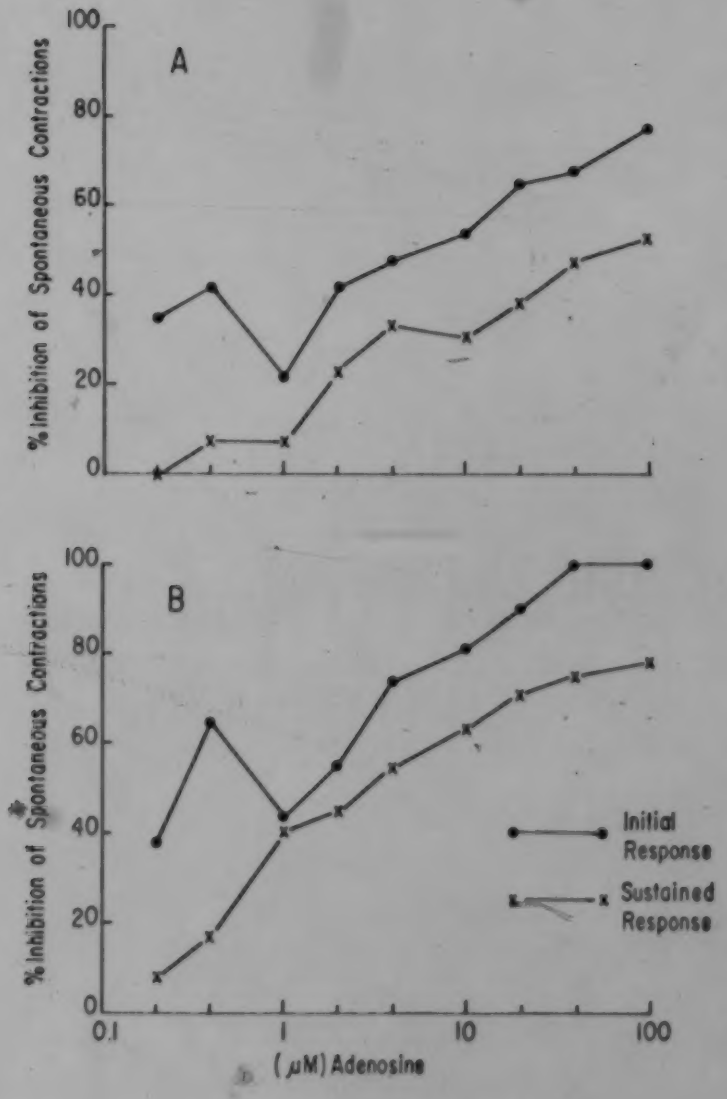


Figure 10: Dose-response curves to adenosine.
A and B illustrate both initial and sustained responses in two strips from a single animal. Responses are dose-dependent between about 0.1 and 100uM.

occurred following washout of high doses but instead a small degree of sensitisation appeared in some strips over 3-4 hours. For this reason successive dose-response curves were not directly comparable.

If a second dose of adenosine was administered in the continued presence of a dose inducing a sustained response, the response to the cumulative dose was less than that to single administration of the same total dose, even when the time interval between responses was as short as 90s (Figure 11). Nevertheless, if large doses were administered to one tissue at 10min intervals with intermediate washing, responses were highly reproducible. This phenomenon is therefore not equivalent to tachyphylaxis, which usually developes and decays more slowly, but resembles antagonism by effective doses of adenosine upon subsequent doses of the same drug. This 'autoinhibition' was not observed when the first dose was ineffective or evoked only an initial response.

Figure 11: Autoinhibition of the adenosine response.
Adenosine (AR) or ATP was administered at the uM concentrations indicated. The response to 40uM adenosine, administered 10min previously, differed from that to the same cumulative dose. 10uM ATP induced only an initial response during autoinhibition to adenosine.

3.1.1.2. Effects of cold storage.

To determine whether adenosine responses might be measured reliably in muscle which had been retained in Tyrode solution at 4°C for several hours following sacrifice of the rabbit, 16 strips were dissected from an animal, 12 of which were immediately stored in the cold. The remaining 4 were mounted in organ baths and responses were elicited in each strip to various doses of adenosine, ATP, epinephrine and tetramethylammonium iodide. The same procedure was followed with groups of 4 strips following 5.5, 11 and 24 hours of cold storage. While epinephrine responses were remarkably similar among groups despite deterioration in the amplitude and frequency of spontaneous contractions in strips stored for 24 hours, responses to adenosine and ATP were appreciably diminished following 11 hours of cold storage. At this time interval, 10uM adenosine elicited no sustained response, and the response to 50uM adenosine was greatly attenuated. Stimulatory responses elicited by tetramethylammonium iodide, which in all cases were virtually abolished by 1mM procaine, deteriorated with an apparently similar time course. It was therefore concluded that responses to adenosine were more labile than those to epinephrine under these storage conditions and the adenosine response was subsequently studied only in freshly dissected strips.

For measurement of tissue cyclic AMP levels, epinephrine and isoproterenol were administered to strips stored at 4°C for up to 3 hours.

The ganglion stimulant tetramethylammonium iodide was included in this experiment to provide an indication of the viability of the nervous components of the tissue. From the qualitative results obtained it was not possible to distinguish between the rates of deterioration of adenosine responses and of nervous activity.

3.1.1.3. Investigation of neural involvement in the adenosine response.

If the integrity of the neural components of the tissue were required for the manifestation of adenosine responses, adenosine might act indirectly by one of two mechanisms:

1) by stimulation of ganglia, followed by propagation of an action potential within the affected fibres and subsequent release of an inhibitory transmitter by the physiological neural mechanisms.
2) by an intervention at nerve terminals promoting release of an inhibitory transmitter without incurring the initiation of action potentials.

The first possibility was investigated using local anaesthetic drugs at doses which selectively depressed generation of action potentials in nerve. The appropriate doses were determined by their capacity to abolish stimulatory responses evoked by tetramethylammonium iodide, without affecting the spontaneous activity of the muscle. Neither 1mM procaine nor 0.17mM lidocaine antagonised responses to adenosine, indicating that adenosine does not possess ganglion stimulant activity. A similar conclusion regarding the adrenergic nervous system in particular was reached when the neurone blocking agent guanethidine failed to antagonise adenosine responses in a Finkelman preparation of the rabbit intestine, while preventing responses to periarterial nerve stimulation.

The gut is innervated by both adrenergic and nonadrenergic inhibitory components of the autonomic nervous system. The possibility that adenosine releases noradrenaline from sympathetic nerve endings within Auerbach's plexus was investigated using rabbits pretreated with 4mg reserpine administered subcutaneously 18 hours before sacrifice, followed by an additional 2mg reserpine intravenously one hour before sacrifice. Since adenosine dose response curves in longitudinal muscle from these animals were similar to those obtained from animals whose noradrenaline stores were not depleted, adenosine

responses may not be attributed to release of noradrenaline. The transmitter at nonadrenergic inhibitory nerve terminals in the gut is currently believed to be ATP or another adenosine nucleotide (Burnstock et al., 1970). Adenosine responses were dissimilar to those induced by histamine, serotonin or prostaglandin E1, confirming that adenosine does not act via the mediation of these substances. Since ATP responses in this muscle were, in all aspects encompassed within this study, qualitatively and quantitatively analogous to adenosine responses (see Section 3.1.1.5), it may be concluded that adenosine relaxes intestinal longitudinal muscle by a direct effect.

3.1.1.4. Influence of drugs on the adenosine response.

Adenosine responses were unaffected by 10uM propranolol but were antagonised by 10uM phentolamine. This effect of phentolamine has been extensively reported in the literature (see Section 3.1.3). In view of the evidence that adenosine does not act by releasing noradrenaline from nerve terminals (Section 3.1.1.3), inhibition of adenosine responses was probably due to a non-specific membrane effect of phentolamine. Since the effect of phentolamine on responses to ATP in this tissue

has been investigated by Weston (1971), no attempt was made in the present study to quantitate this effect.

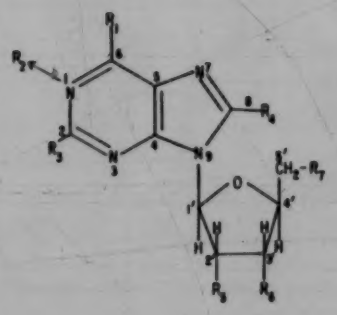
Adenosine responses were not appreciably modified by 10uM atropine or by 0.1-5uM papaverine, 2-10uM 1-methyl,3-isobutylxanthine or 10-20uM imidazole. These observations are not consistent with the hypothesis that adenosine responses are mediated by cyclic AMP since, if such were the case, the phosphodiesterase inhibitors papaverine and methylisobutylxanthine should potentiate responses, while the phosphodiesterase stimulant imidazole should antagonise them.

Effects of dipyridamole and theophylline are reported in Sections 3.1.1.5.2 and 3.1.1.6, respectively.

3.1.1.5. Adenosine analogues.

3.1.1.5.1. Responses to analogues in longitudinal muscle.

Adenosine-like responses were induced by purine ribosides with a primary amino group (as in adenosine) or a secondary amino group in the 6-position (Figure 12). The only exception encountered was an 8-substituted derivative, 8-bromoadenosine, which was inactive.



Substance	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Relaxant Activity
Adenosine	NH_2	-	H	H	OH	OH	OH	*
AMP	NH_2	-	H	H	OH	OH	PO_4^{2-}	*
ADP	NH_2	-	H	H	OH	OH	$P_2O_7^{3-}$	*
ATP	NH_2	-	H	H	OH	OH	$P_3O_{10}^{4-}$	*
α,β-Methylene ADP	NH_2	-	H	H	OH	OH	$PO_3CH_2PO_3^{3-}$	+*
N^6-Hydroxyadenosine	NHOH	-	H	H	OH	OH	OH	*
N^6-Methyladenosine	$NHCH_3$	-	H	H	OH	OH	OH	*
N^6-Isopentenyladenosine	NHC_5H_9	-	H	H	OH	OH	OH	*
Adenosine-N^1-oxide	NH_2	O	H	H	OH	OH	OH	*
2'-Deoxyadenosine	NH_2	-	H	H	H	OH	OH	±
3'-Deoxyadenosine	NH_2	-	H	H	OH	H	OH	±
Inosine	OH	-	H	H	OH	OH	OH	±
8-Bromoinosine	OH	-	H	Br	OH	OH	OH	±
8-Bromoguanosine	OH	-	NH_2	Br	OH	OH	OH	±
8-Bromoadenosine	NH_2	-	H	Br	OH	OH	OH	-
6-Mercapto-PRR	SH	-	H	H	OH	OH	OH	-
6-Methyl-PRR	CH_3	-	H	H	OH	OH	OH	-
6-Methylmercapto-PRR	SCH_3	-	H	H	OH	OH	OH	-
6-Methoxy-PRR	OCH_3	-	H	H	OH	OH	OH	-
6-Dimethylamino-PRR	CH_3NCH_3	-	H	H	OH	OH	OH	-
6-Chloro-PRR	Cl	-	H	H	OH	OH	OH	-
2-Amino,6-Chloro-PRR	Cl	-	NH_2	H	OH	OH	OH	-

Figure 12: Adenosine analogues employed in this study.
Relaxant activity: +: approximately equiactive with adenosine; *: adenosine-like activity below 10uM, see text; ±: less potent than adenosine by at least three orders of magnitude: -:no adenosine-like activity.

Analogues found to elicit adenosine-like responses were ATP, ADP, AMP, alpha,beta-methylene adenosine diphosphate, cyclic AMP, N^6-hydroxyadenosine, adenosine-N^1-oxide, N^6-methyladenosine and N^6-isopentenyladenosine.

Analogues in which the ribose moiety was missing (adenine) or substituted by deoxyribose (2'-deoxyadenosine and 3'-deoxyadenosine) induced at 10-100uM only small, very transient initial responses. Since initial responses appear at lower doses of adenosine than do sustained responses, it is possible that the responses observed with these analogues represented the extreme lower end of a dose-response curve resembling that to adenosine. If this be true, these analogues must be less potent than adenosine by at least three orders of magnitude. Other analogues which induced only small, transient responses at high doses were inosine, 8-bromoguanosine and 8-bromoinosine.

Analogues which demonstrated no adenosine-like activity at 100uM were purine riboside, its 6-mercapto-, 6-methyl-, 6-methylmercapto-, 6-methoxy-, 6-dimethylamino-, 6-chloro- and 2-amino,6-chloro-substituted derivatives, and 8-bromoadenosine.

3.1.1.5.2. Comparison of active analogues.

The relaxant responses to the adenosine nucleotides ATP, ADP and AMP were compared to those of adenosine. Qualitatively, the response patterns were indistinguishable. Consecutive dose-response curves performed between pairs of these four substances in rotating orders further indicated that all four were roughly equiactive. When ATP and adenosine were compared between 1 and 80uM by administering each drug alternately with intermediate washing, their dose-response curves coincided (Figure 13). ATP responsiveness was found to be influenced by cold storage and drugs in a manner identical to adenosine and mutual antagonism was manifested between sustained responses to ATP and adenosine, as it was between those to other adenosine-like drugs and adenosine or ATP.

These observations raise the question of whether the adenine nucleotides exert their effects per se or only subsequent to enzymatic degradation by ubiquitous, extracellular phosphorolytic enzymes to adenosine itself. This point is also important in attempting to assign a site of action to adenosine since the nucleoside can freely permeate cell membranes while nucleotides generally cannot. That these permeation characteristics also apply

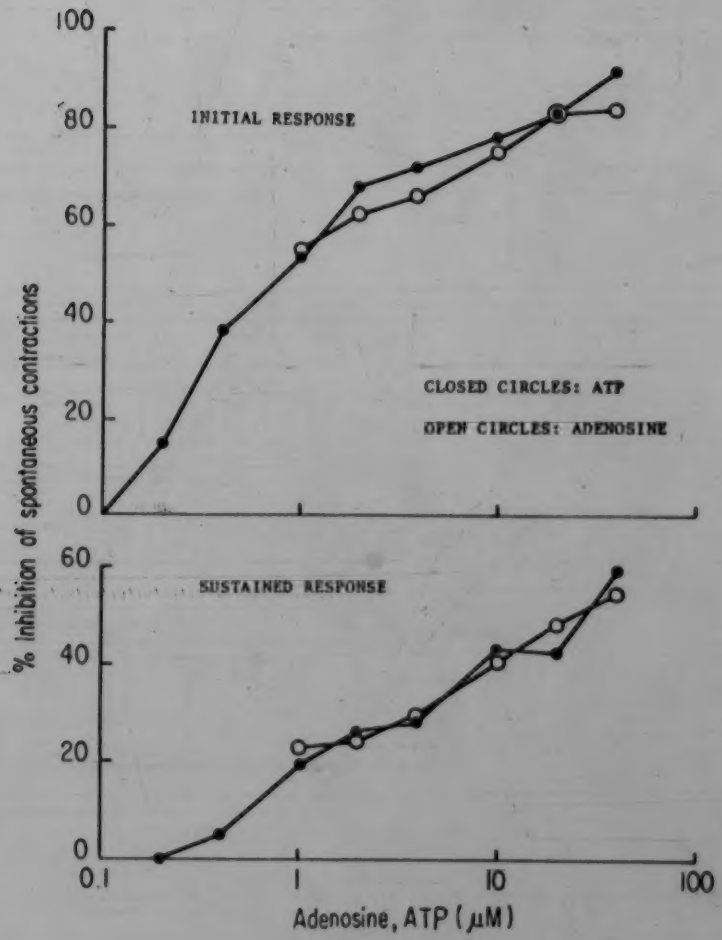


Figure 13: Dose-response curves to adenosine and ATP.
Curves obtained when ATP and adenosine were administered alternately.

to gut (taenia coli) was indicated by the work of Lynch (Burnstock, 1972). Since responses to nucleotides in intestinal longitudinal muscle are of equal magnitude and rapidity as those to adenosine, enzymatic breakdown due to tissue phosphatases, ATPase and nucleotidase would have to be complete within very few seconds if relaxant activity is to be ascribed to adenosine alone.

This problem was investigated by the use of specific pharmacological tools. Nitrobenzylthioguanosine inhibits purine nucleoside transport in human erythrocytes by 50% at around 10uM (Paterson & Oliver, 1971 and Paul et al., 1975) and also inhibits adenosine transport in HeLa cells (Paterson & Babb, personal communication), canine heart (Olsson et al., 1973) and guinea-pig cerebral cortical slices (Huang & Daly, 1974). It was assumed that this compound would also inhibit adenosine entry into smooth muscle cells, thus modifying the response if the site of action was intracellular. At 10uM this compound had no effect, however, on responses to adenosine or ATP, suggesting that nucleoside transport is neither essential for production of responses nor important in their termination in this tissue. Similar results were obtained using 1-4uM dipyridamole, a compound known as an inhibitor of facilitated transport of adenosine and also as an inhibitor of adenosine deaminase.

The ADP analogue alpha,beta-methylene-ADP is an extremely potent inhibitor of 5'-nucleotidase at doses as low as 0.1uM (Burger & Lowenstein, 1970 and Baer & Simonson, 1975). Since its phosphonate group is not amenable to enzymatic hydrolysis, this nucleotide is unlikely to be degraded to adenosine or transported into the cell within a period of seconds. Nevertheless, this compound not only failed to antagonise responses to adenosine and its nucleotides, indicating that nucleotidase activity is not necessary for nucleotide responses, but also itself induced adenosine-like responses below 10uM. Since alpha,beta-methylene-ADP failed to antagonise adenosine responses while itself inducing an adenosine-like response, and since it also could not be expected to enter cells within the period of observation, it is possible that 'autoinhibition', rather than the response to adenosine, is a result of adenosine entry into cells. This interesting question has not been further explored in this study. The above results are consistent with a common extracellular site of action for adenosine and its nucleotides.

It may be of interest to mention that while alpha,beta-methylene-ADP induced adenosine-like responses at doses below 10uM, it manifested an additional dose-

dependent effect at higher concentrations. This consisted of an increase in the amplitude of spontaneous contractions and could be particularly well observed using cumulative doses. As shown in Figure 14, with accumulating doses of alpha,beta-methylene-ADP, the adenosine-like response persisted but was overcome more rapidly at higher doses by the additional stimulatory effect of this analogue. The effect of each total dose was similar whether administered singly or cumulatively.

Cyclic AMP evoked responses in many ways similar to those of adenosine. The transient response was, however, generally less pronounced in relation to the sustained response than was the case with other nucleotides and achieved a maximal value only after about 30s. From the appearance of responses and the fact that the moieties necessary for adenosine-like activity are present in the cyclic AMP molecule, it seems likely that at least part of the response to cyclic AMP in this tissue is due to adenosine-like activity. The only factor which might reduce the affinity of cyclic AMP for the site of action of adenosine and its nucleotides would be distortion of the ribose ring or the position and function of its 3'-oxygen due to cyclisation of the phosphate group.

The substituted adenosine nucleosides, N^6-

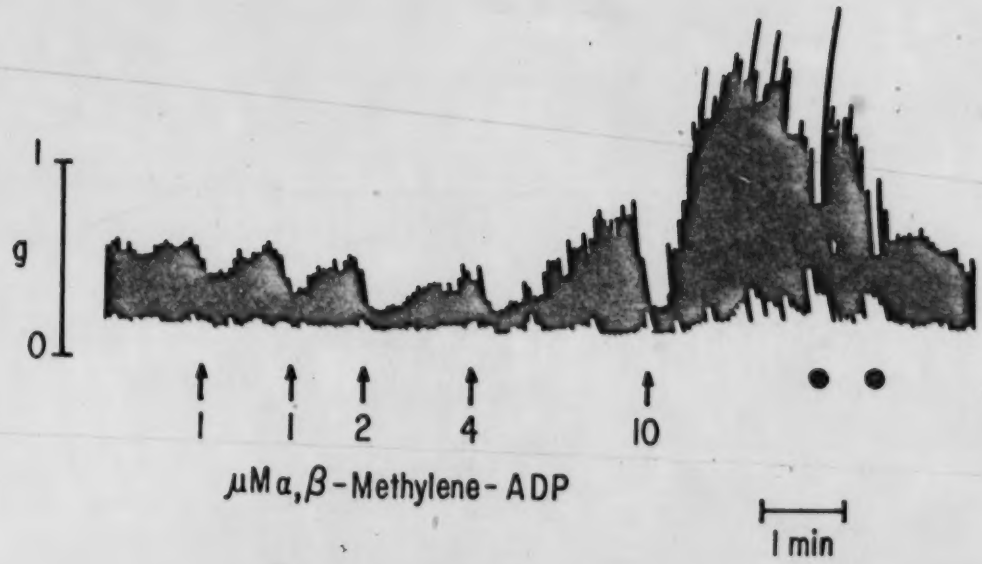


Figure 14: Cumulative responses to alpha,beta-methylene-ADP. Between cumulative doses of 1 and 4uM, adenosine-like inhibition of spontaneous contractions was observed. At 8 and 18uM, dose-dependent stimulation of spontaneous activity predominated. Responses were indistinguishable whether administered singly or cumulatively. The bathing fluid was changed at •.

hydroxyadenosine, N^6-methyladenosine, adenosine-N^1-oxide and N^6-isopentenyladenosine were approximately equiactive with adenosine.

In summary, the molecular specifications required for adenosine-like activity are rather strict, although they encompass several naturally occuring substances. To the basic adenosine molecule may be added substituents at various sites, excluding the 8-position, without appreciable change in activity (see Figure 12). The substitution of one hydrogen at the 6-amino group is permissible, but double substitution or elimination of the amino group leads to total loss of activity. It would appear that the N^6-amine function is involved in hydrogen bond formation at the receptor binding site. Omission of the ribose moiety, or replacement with 2'- or 3'-deoxyribose also leads to virtual loss of activity.

3.1.1.6. Antagonism of adenosine responses.

Investigation of the mechanism of action of adenosine has been hindered particularly by the lack of a competitive antagonist for adenosine responses. For this reason, a limited effort was made to screen a series of available adenosine analogues, in particular those lacking

agonistic properties, for antagonism of adenosine responses. This was performed by comparing responses to adenosine immediately before and after administration of a 100uM dose of each analogue. Of sixteen analogues screened in this manner, only those possessing adenosine-like activity modified adenosine responses due to the phenomenon of 'autoinhibition' discussed in Sections 3.1.1.1 and 3.1.1.5.2. It is of interest that neither purine riboside nor its 6-chloro-, 2-amino,6-chloro- or 6-methoxy-substituted derivatives, which inhibit adenosine deamination, modified the sustained response, indicating that the biphasic effect is not due to deamination of adenosine by the tissue.

The only purine which was found to antagonise adenosine responses without itself inhibiting spontaneous activity was theophylline, whose structure is shown in Figure 15. Methylisobutylxanthine did not antagonise adenosine responses. The mean response to 100uM theophylline itself over 18 experiments was an increase in the amplitude of spontaneous contractions of 8 ± 3% (range -23 to +33%), which, although small, is significant at the 5% level. This dose of theophylline, which potentiated epinephrine responses (Section 3.1.2), antagonised the response to 10uM adenosine by 68.3 ± 4.9%. It was also consistently observed that high doses of adenosine (above

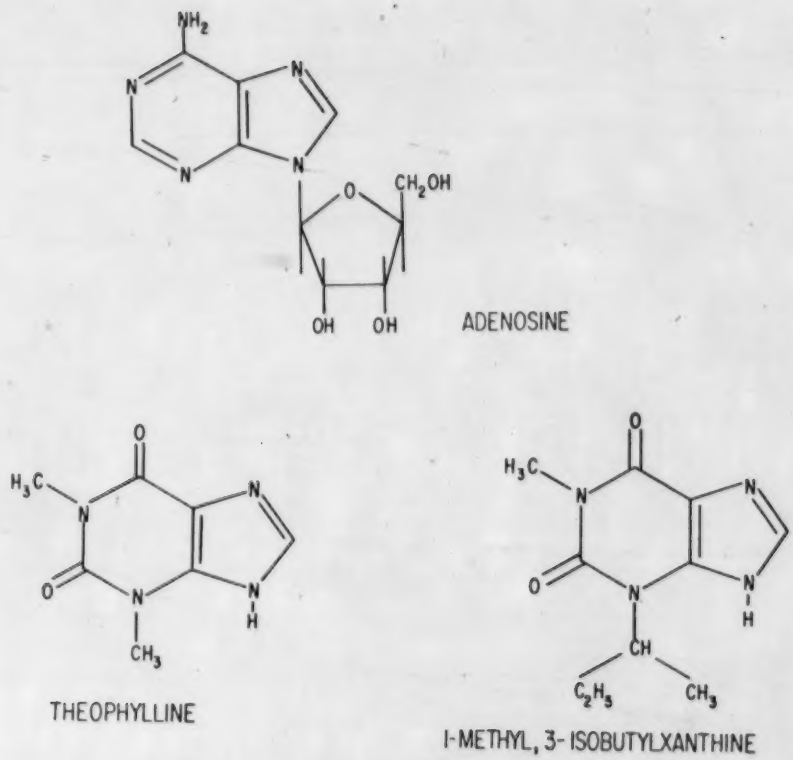


Figure 15: Structures of adenosine, theophylline and methylisobutyl-xanthine.

100uM) evoked responses in the presence of theophylline which were similar to the maximum response inducible in the tissue before theophylline treatment, indicating that the antagonism due to 100uM theophylline was surmountable.

3.1.2. Sympathomimetics.

Since spontaneous activity of intestinal muscle is inhibited by both alpha- and beta-adrenergic mechanisms, responses to phenylephrine, isoproterenol and epinephrine were compared to determine qualitative differences between responses evoked by a predominantly alpha- or beta-adrenergic or a mixed adrenergic agonist, respectively, and to compare these to adenosine responses. As shown in Figure 3, epinephrine evoked a rapid initial response which was maximal in 10-15 seconds and which waned to a smaller, sustained response within 1 minute. Responses to phenylephrine were similar to those of epinephrine. Isoproterenol, however, induced a slower response which was usually maximal within 1 minute (Figure 3). When responses at 1 minute were measured, dose-response curves to epinephrine and isoproterenol fell within approximately 0.01-2uM.

Responses to 0.1uM epinephrine were partially

antagonised by 10uM propranolol and more markedly antagonised or abolished by 10uM phentolamine, indicating that epinephrine acted in this muscle predominantly on alpha- but also on beta-adrenergic receptors. The likelihood of partial beta-receptor involvement in epinephrine responses was also indicated when 100uM theophylline was found to potentiate sustained responses to 0.05uM epinephrine by 28 ± 7%, compared to 43 ± 8% potentiation of 0.05uM isoproterenol, based on six experiments each. The adenosine response strikingly resembled that to epinephrine, but could not be attributed to catecholamine release for the reasons discussed in Section 3.1.1.3. It is, however, possible that adenosine affects smooth muscle tone via two receptor systems or pathways, as does epinephrine. In the absence of any specific adenosine antagonists this possibility could not be excluded and epinephrine was included in cyclic AMP determinations as a control drug.

3.1.3. Discussion of relaxant responses.

The responses to adenosine in the longitudinal muscle of the rabbit intestine described in Section 3.1.1.1 are similar to those described by several workers using isolated rabbit small intestine (Bishop et al., 1963; Kim

et al., 1968 and Bowman & Hall, 1970). The transient nature of the responses which was emphasised by Bowman & Hall might have been less evident had they allowed drugs to remain in contact with the tissue for more than 1 minute. In the current studies adenosine and its analogues induced sustained responses which were usually achieved within one minute but which could not have been identified had drugs not been left in contact with the tissue for 2 minutes. In the present study, responses were better sustained than those described in longitudinal muscle by Weston (1971 and 1973b), who found recovery to 90% of control amplitude within 15min of administration of 100uM ATP. Such transient responses could not be reproduced in the present study even in the presence of 10uM atropine, as routinely used by Weston. It is possible that this discrepancy may be due to the fact that Weston used physiological saline solution containing 2.5mM calcium, as opposed to our 1.9mM. Since sustained responses were reproducible and dose-dependent and, as with epinephrine, appeared to constitute the predominant response, the effects of pharmacological agents on this phase of the response have been studied and reported above. The only qualitative difference observed between the transient and the sustained phases of the response was that 'autoinhibition' did not always affect both to the same degree. It should therefore be borne in mind that

transient and sustained responses may not be manifestations of the same drug-receptor interaction or molecular mechanisms.

The finding that adenosine responses deteriorated relatively rapidly when muscle was stored at 4°C confirms the observation of McDougal & Borowitz (1972) utilising guinea-pig ileum. Since cold storage for such a brief period (< 24h) is unlikely to induce specific impairment of nervous activity, however, this does not constitute firm supportive evidence for an indirect effect of adenosine via neural elements. On the contrary, the experiments using local anaesthetics, guanethidine and reserpine confirm the conclusion of Burnstock et al. (1970) that adenosine acts directly on smooth muscle, provided that an as yet unknown substance is not the true 'purinergic' transmitter and that ATP and adenosine do not release it from non-adrenergic inhibitory nerve terminals.

The phenomenon of 'autoinhibition' has been observed by others at 100uM ATP or adenosine in rabbit ileum (Mihich et al., 1954 and Kim et al., 1968) rabbit longitudinal muscle (Weston, 1973b) and guinea-pig ileum (Weston, 1973a) but not in guinea-pig taenia coli (Burnstock et al., 1970) and has erroneously been described as tachyphylaxis. Rather, it resembles the

'autoinhibition' of alpha-mediated epinephrine-induced contractions in rat vas deferens (Barnett et al., 1968), rat uterus (Triner et al., 1970) and vascular smooth muscle (Somlyo & Woo, 1967 and Guimaraes, 1972). Since Somlyo & Woo (1967) and Triner et al. (1970) found this effect of epinephrine to be prevented by propranolol, they concluded that 'autoinhibition' was a result of a beta-receptor mediated increase in cyclic AMP. By analogy, it is possible that 'autoinhibition' of adenosine responses is a manifestation of opposing influences of adenosine on the muscle. In the present study 'autoinhibition' was observed at any effective dose and was evident within 90s of drug addition. Reversal of this effect was also considerably more rapid than has been reported by Weston, who washed the tissue for 45min. The findings reported above with alpha,beta-methylene-ADP suggest that this phenomenon is not due to desensitisation of the receptor complex but rather to another event, possibly based on an intracellular effect of adenosine. This hypothesis readily lends itself to further investigation.

All results reported in Section 3.1.1 are consistent with a common extracellular site of action for adenosine and its nucleotides. A similar conclusion has been reached by Axelsson & Holmberg (1969) based on the inability of inhibitors of ATPase to antagonise responses

to ATP in guinea-pig taenia coli.

Conflicting reports appear in the literature concerning the effects of dipyridamole and imidazole on adenosine responses in intestinal smooth muscle. Whereas Stafford (1966) and Satchell et al. (1972) observed potentiation, Hulme & Weston (1974a) detected no significant shift in adenosine dose-response curves in rabbit duodenum with 0.01-1uM dipyridamole. Since 1uM dipyridamole is sufficient to inhibit adenosine uptake (Hulme & Weston, 1974b) and reduced the amplitude of spontaneous contractions by about 30% in the present studies, the relevance of any apparent effect on adenosine responses at higher doses would be doubtful due to tension changes. The lack of effect at 1uM suggests that adenosine entry into cells is unimportant in terminating responses (Hulme & Weston, 1974a) or in facilitating them.

Imidazole has been reported to antagonise ATP responses at a concentration of approximately 50mM in guinea-pig taenia coli (Rikimaru et al., 1971; Tomita & Watanabe, 1973 and Satchell & Dann, unpublished) and at 75mM but not at 50mM in rabbit intestine (Bowmann & Hall, 1970). These doses are extremely high in consideration of the fact that in the current studies 20uM imidazole increased the amplitude of spontaneous contractions by

about 40%. As suggested by the latter workers, effects at such doses are probably nonspecific. If imidazole indeed contracts smooth muscle by stimulating phosphodiesterase and thus decreasing tissue cyclic AMP levels, as suggested by Andersson (1973c), the optimal dose for observation of antagonism of a cyclic AMP mediated response should be 10-20uM since in this range changes in tissue tension are minimal. No such antagonism was observed in this study.

Antagonism of adenosine and ATP responses in intestinal muscle by phentolamine has been reported by other workers. Phentolamine at 180-350uM almost abolished the inhibitory potential and response caused by 1-10uM ATP in guinea-pig taenia coli (Rikimaru et al., 1971; Burnstock, 1972 and Tomita & Watanabe, 1973) but inhibition could be overcome by higher doses of ATP. 2.5-3.5uM Phentolamine has, however, been shown to antagonise responses to phenylephrine but not to adenosine or ATP in guinea-pig taenia coli (Tomita & Watanabe, 1973), rabbit intestine (Kim et al., 1968 and Bowman & Hall, 1970) and rabbit intestinal longitudinal muscle (Weston, 1971). In the present studies 10uM phentolamine was found to antagonise responses to 40uM adenosine or ATP by about 30%. Since Weston quotes the pA10 value for phentolamine versus phenylephrine in this tissue as 6.7, it is unlikely that alpha-adrenergic receptors are involved in the

adenosine response.

The methylxanthine phosphodiesterase inhibitors theophylline and 1-methyl,3-isobutylxanthine had divergent effects on adenosine responses which have not hitherto been reported. In contrast to the observations of Bowman & Hall (1970) using rabbit intestine, 100uM theophylline did not significantly alter the frequency of spontaneous contractions and stimulated their amplitude minimally but significantly (8 ± 3%). Higher concentrations inhibited both. This dose of theophylline also invariably antagonised responses to adenosine or ATP while potentiating those to epinephrine or isoproterenol, in contrast to the diverse effects observed by these workers with 100-1000uM theophylline. It is possible that, by ignoring the sustained component of drug responses, the difference between the theophylline effect on catecholamines and on adenosine and ATP may have been obscured in their system. Alternatively, the sensitivity of isolated whole intestine to theophylline may be more variable than longitudinal muscle and consequently a dose capable of modifying drug responses without prejudicing spontaneous activity may not have been identifiable in the study by Bowman & Hall. It is interesting to speculate whether in the present study 100uM theophylline stimulated spontaneous activity in intestinal muscle by a mechanism

similar to the calcium-mobilising action of caffeine reported in vascular muscle by Somlyo & Somlyo (1968).

Antagonism of adenosine responses was not shared by methylisobutylxanthine, an analogue of theophylline (see Figure 15). In six separate experiments responses to 100uM adenosine after 10uM methylisobutylxanthine were 114.7 ± 5.5% of their respective controls. Since this dose of methylisobutylxanthine, a more potent phosphodiesterase inhibitor than theophylline, reduced the amplitude of spontaneous contractions by 45.5 ± 5.5%, phosphodiesterase inhibition seems to have no influence of functional significance on adenosine responses and, on the other hand, no antagonism between methylisobutylxanthine and adenosine seems to exist. Responses to 10uM adenosine or ATP were unaffected by 2-5uM methylisobutylxanthine. These results contrast with evidence of antagonism by both theophylline and methylisobutylxanthine of adenosine-induced cyclic AMP accumulation in guinea-pig cerebral cortical slices (Huang et al., 1972). In the latter instance, antagonism by theophylline has been suggested to be competitive. In intestinal longitudinal muscle the antagonism induced by 100uM theophylline upon responses to adenosine and ATP may also be overcome by high doses of agonist (Section 3.1.1.6). Since theophylline demonstrates a number of pharmacological responses,

possibly due either to its ability to inhibit cyclic nucleotide phosphodiesterase or to some direct effects on calcium movements, this feature of its antagonism of adenosine responses in smooth muscle does not indicate that the antagonism is necessarily competitive. In view of the fact that neither nitrobenzylthioguanosine nor dipyidamole antagonised responses to adenosine, although they inhibit adenosine transport in other systems more effectively than does theophylline, it is unlikely that antagonism by theophylline was due to impairment of adenosine uptake into cells. Nevertheless, since the hypothesis to be tested was that smooth muscle relaxation induced by these substances may be mediated by cyclic AMP, theophylline was chosen, in the absence of a reliable competitive antagonist, as the drug most likely to interfere with a possible stimulatory effect of adenosine on smooth muscle adenylate cyclase, choosing doses which do not affect cyclic AMP levels by themselves.

Structure-activity relationships of adenosine analogues in nicotine-stimulated longitudinal muscle of the guinea-pig ileum have been found by Leslie et al. (1973) to be somewhat different from those in vascular smooth muscle (see Section 1.1.4). Guinea-pig ileal longitudinal muscle was more responsive than aortic strips to analogues in which the electron distribution in the

purine ring was altered (1-methyladenosine and toyocamycin) whereas ileal muscle was less responsive than aorta to 2'-deoxyribosides (2'-deoxyadenosine and 2',3'-isopropylidene adenosine). Both types of smooth muscle exhibited requirements for hydrogen bonding at the N^6-position and for basicity at the N^1-position. The results reported in Section 3.1.1.5.1 are in agreement with the necessity for hydrogen bonding in intestinal muscle at the N^6 and 2'-positions as proposed by these workers and further suggest that the 3'-position constitutes another such site. In rabbit as opposed to guinea-pig intestinal longitudinal muscle, however, adenosine-N^1-oxide elicited adenosine-like responses, indicating that a species difference exists over the importance of the N^1-position as a nucleophilic site. The inability of 8-bromoadenosine to induce a response corresponds with the inability of this analogue to bind to the protein carrier for adenosine transport in myocardium (Olsson et al., 1973). Adenosine deaminase also will not utilise several 8-substituted adenosines as substrate (Simon et al., 1970). It is likely that substitutions at this site prejudice the free rotation of the purine relative to the ribose ring which in this case prevents the adoption of the conformation about the glycosidic bond appropriate for either enzyme catalytic (Hampton et al., 1972) or drug receptor sites (Olsson et al., 1973).

All other things being equal, the relative potencies of adenosine and its nucleotides might be expected to vary in different test systems according to the relative speed with which their responses are terminated. In intact animals, adenosine responses are very rapidly terminated by uptake into tissues, especially lung, and subsequent phosphorylation or deamination, while nucleotides would first be dephosphorylated to adenosine. In isolated tissues, however, these processes usually become less influential on responses due to the relatively large reservoir of extracellular adenosine in the organ bath. Nevertheless, although Burnstock et al. (1970) and Axelsson & Holmberg (1969) found responses to ATP and ADP in guinea-pig taenia coli to fall in a dose range similar to the present study, AMP and adenosine exhibited only one hundredth of the potency of the other nucleotides in that tissue. Bowman & Hall (1970) also found the order of potency in rabbit intestine to be ATP > ADP > AMP > adenosine, as would be expected if responses were influenced by uptake and metabolism of adenosine. It may not even be possible to reduce uptake appreciably using transport inhibitors, since at concentrations above 10uM adenosine may permeate cells predominantly by simple diffusion. Differences in the activity of tissue phosphorolytic enzymes, in the volume of the extracellular

drug reservoir in relation to intracellular space, and in the concentration of adenosine or ATP studied may therefore account for the conflicting reports of the effect of dipyridamole on responses to adenosine and nucleotides in isolated smooth muscle. Entry into cells may also account for the failure of adenosine to produce responses equivalent to those of nucleotides in some systems. There is evidence to indicate that under the circumstances of the present study neither nucleotidase activity, uptake nor deamination influences adenosine responses in rabbit intestinal longitudinal muscle. The corollary to this finding is that the true relative potencies of adenosine and its nucleotides may be reflected in this tissue. Although Weston (1973b) concluded that ATP was more potent than adenosine from mean dose response curves compiled for each drug on different strips of rabbit duodenal longitudinal muscle, the standard errors for all points but one on his mean dose response curves overlapped. It is therefore unlikely that a real difference in potency existed. The present study indicated that no difference in potency could be detected between adenosine and its nucleotides on the same strips and that adenosine and ATP were equipotent over a considerable dose range when administered alternately (Figure 13).

3.2. Effects of adenosine on adenylate cyclase.

3.2.1. Characteristics and kinetics of adenosine inhibition.

Adenylate cyclase was prepared from several tissues as described in Section 2.2.1, and was found in all cases to be inhibited by concentrations of 0.01mM adenosine or more. At 1mM, adenosine inhibited cyclase activities by 40 to 80% and fluoride- or hormone-stimulated activities were affected at least as much as basal activity (Table 3). In no tissue was significant stimulation of cyclase by adenosine observed. Since rat brain yields an adenylate cyclase of high specific activity allowing more reliable kinetic measurements, the kinetics of the adenosine effect was investigated using this tissue. The major conclusions were subsequently reexamined using other tissues and appeared to be generally valid. The dose effect curve for adenosine inhibition of adenylate cyclase from rat brain in the presence and absence of fluoride is shown in Figure 16.

Enzyme source	Addition	(pmoles/mg.min) No AR[1]	1mM AR[1]	% Inhibition
Whole rat brain	-	113±1	62±3	41
"	10mM NaF	275±3	105±4	62
Rat cerebellum	-	149±6	60±3	60
Rat cerebral cortex	-	50±2	29±1	42
Rat kidney cortex	10mM NaF	62±3	28±1	55
Ehrlich ascites	-	10.6±0.2	6.1±0.3	42
"	10mM NaF	72±6	25±1	66
"	0.1mM Epi[2]	32±2	12.5±0.2	61
Rat fat cell	-	59±3	27±1	54
"	10mM NaF	414±14	87±1	79
"	1mM Epi[2]	337±2	58±4	83
"	20ug/ml ACTH	187	31±2	83
"	10ug/ml Gluc[3]	146±5	33±2	78
Dog platelets	-	32±2	8.3±0.3	74
"	10mM NaF	163±8	39±3	76
Guinea-pig lung	10mM NaF	165±2	53±2	68
Mouse heart	-	8.5±0.1	3.5±0.4	60
"	10mM NaF	49.6±1.1	12.3±0.1	76
"	0.1mM Epi[2]	9.5±0.1	2.7±0.5	72
Rat heart	-	8.6±0.2	4.2±0.1	53
"	10mM NaF	34.5±1.0	10.9±1.5	68
"	0.1mM Epi[2]	11.8±0.2	5.3±0.4	55
Rabbit heart	-	32.5±1.7	8.8±0.9	73
"	10mM NaF	93.1±2.0	20.6±0.3	78
"	0.1mM Epi[2]	45.1±5.0	10.4±0.8	79
Guinea-pig heart	-	15.1±0.1	5.7±0.1	63
"	10mM NaF	52.8±2.8	13.1±1.5	75
"	0.1mM Epi[2]	20.2±0.5	9.4±0.7	56
Rabbit intestinal muscle	10mM NaF	109±8	32±4	71

Table 3: Effect of 1mM adenosine on adenylate cyclases.
1: adenosine; 2: epinephrine; 3: glucagon.

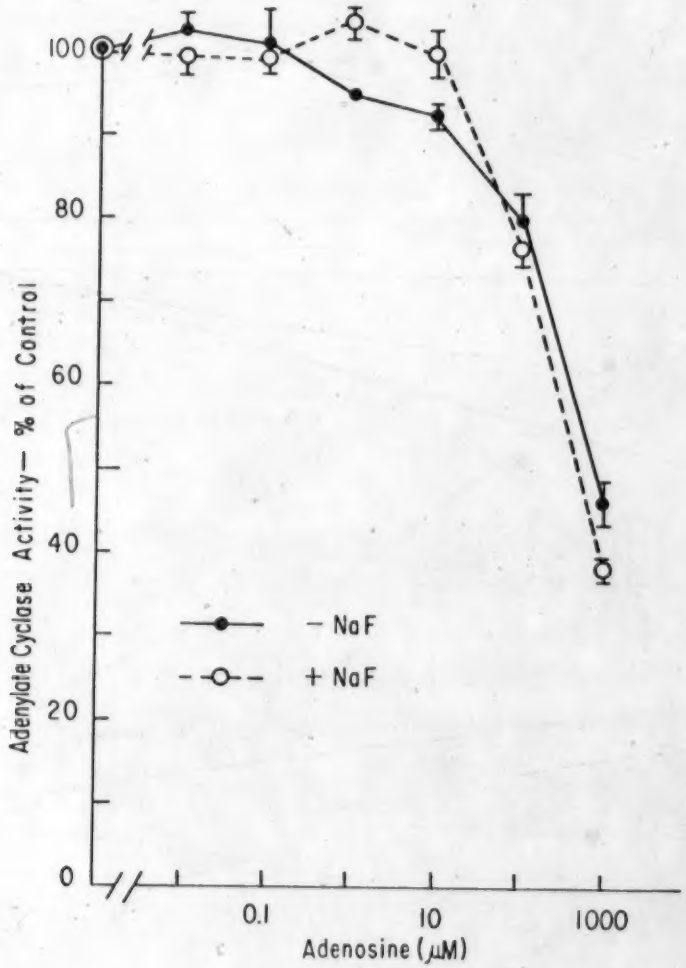


Figure 16: Dose-effect curve of adenosine on adenylate cyclase from rat brain.
Curves in the absence and presence of 10mM sodium fluoride. Means ± standard error of triplicate determinations. 100% in the presence of fluoride was 274 ± 3pmoles/mg.min and in its absence 163 ± 3pmoles/mg.min.

3.2.1.1. Time dependence and reversibility.

When rat brain cyclase was added to complete reaction mixtures, cyclic AMP production as a function of time was found to be linear in the absence and presence of 2mM adenosine (Figure 17). This indicated that inhibition by the nucleoside was essentially immediate following addition of enzyme. Coincident rates of cyclic AMP formation were also observed in control incubations and in tubes in which the enzyme had been exposed to 2.5mM adenosine for 1min followed by addition of adenosine deaminase and ATP as substrate. These observations were further confirmed by incubating the enzyme with 2.5mM adenosine for 1min prior to initiation of the reaction with ATP. The inhibitory effect of adenosine was immediately reversed after 5min by addition of sufficient adenosine deaminase to hydrolyse all adenosine present to inosine within 15s (Figure 18). Similar observations were made using cyclase from Ehrlich ascites and rat fat cells. These results indicate that the effect was due to adenosine per se and was not artifactual, for example due to phosphorylation of adenosine by any contaminating nucleoside kinase and myokinase in the cyclase preparation and subsequent dilution of the ATP-alpha-^{32}P substrate of the cyclase reaction.

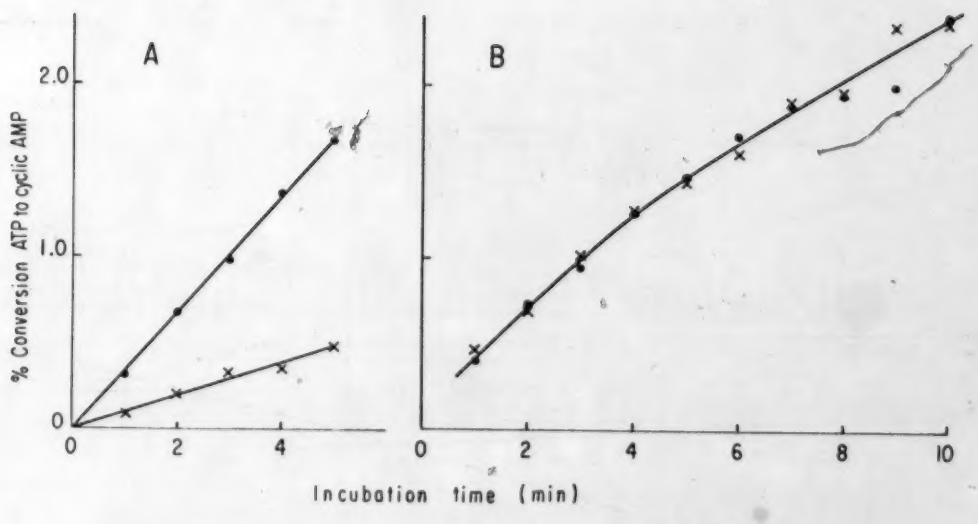


Figure 17: Time dependence and reversibility of adenosine-induced inhibition of cyclase.
Samples were withdrawn from incubation media at the appropriate times and immediately spotted over EDTA-stop solution on PEI plates. Dots represent individual determinations from control tubes, crosses represent determinations from media exposed to adenosine. A: in the continued presence of 2mM adenosine. The reaction was initiated by addition of enzyme. B: adenosine deaminase added after exposure of the enzyme to 2.5mM adenosine for one minute. The reaction was initiated 15s later by addition of substrate.

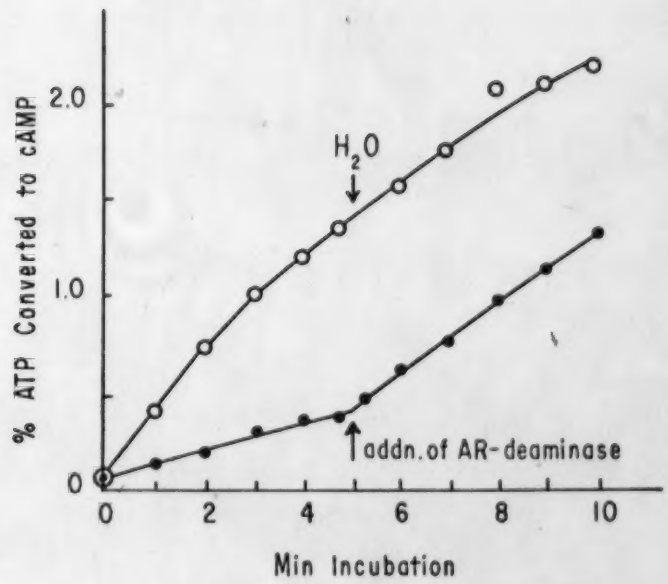


Figure 18: Reversal of adenosine-induced inhibition of cyclase. The reaction was initiated by addition of substrate. Open circles: adenosine and adenosine deaminase were added before the enzyme. Water added 5min after initiation of the reaction. Closed circles: adenosine in contact with the enzyme for 1min before initiation of the reaction. Inhibition was immediately reversed upon addition of adenosine deaminase at 5min.

3.2.1.2. Kinetics.

The kinetics of adenosine inhibition of adenylate cyclase was studied by comparing cyclase activity at six concentrations of ATP in the presence of 0, 0.1 and 0.3mM adenosine. Rate curves were obtained in the presence and absence of sodium fluoride and rate parameters with their standard errors were calculated using the FORTRAN computer programme HYPER by Cleland (1963a) which makes a weighted fit to the reciprocal form of the rate equation:

$$V = Vmax\ S\ /\ (Km + S).$$

Figure 19 shows a double reciprocal plot of rate versus substrate concentration with lines fitted by this programme. Calculated values of Vmax and Km with their standard errors are shown in Table 4 for five experiments using two different preparations of rat brain cyclase. By inspection of this table it can be seen that both the slope and the intercept on the Y-axis (Vmax) appear to have been influenced dose dependently by adenosine, although a substantial error exists in the slope estimates, and that the Km for ATP was relatively unaffected. Inhibition by adenosine was therefore clearly non-competitive and the data were subsequently fitted to the equation for linear non-competitive inhibition:

$$V = Vmax\ S\ /\ [\ Km(1 + I/Kis) + S(1 + I/Kii)\ ]$$

using the FORTRAN programme NONCOMP by Cleland (1963a), as

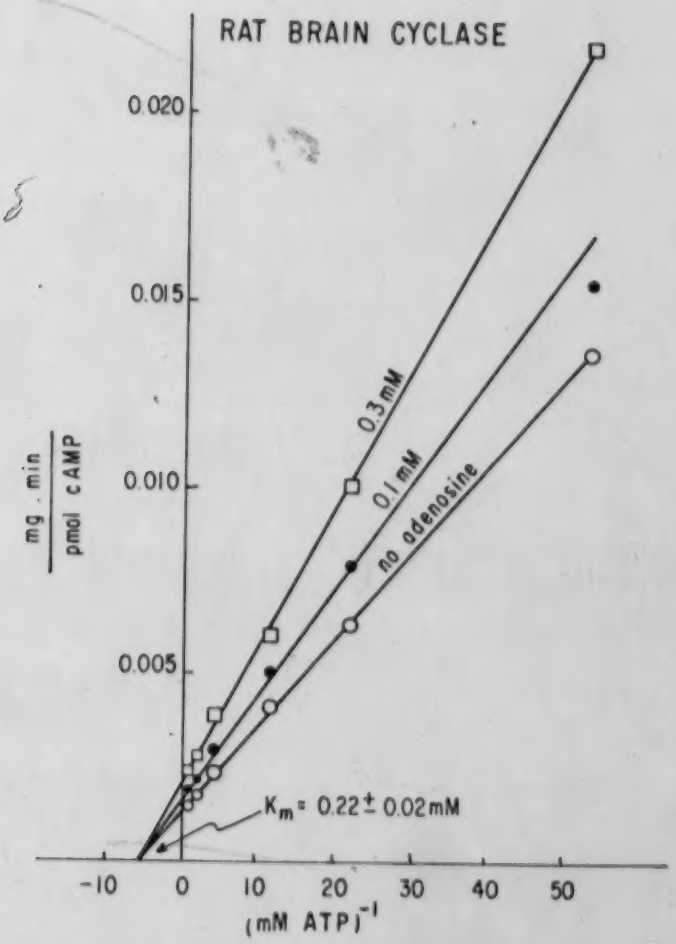


Figure 19: Double reciprocal plot of rate of cyclase activity versus substrate concentration at three concentrations of adenosine. Rat brain cyclase. Points are means of triplicate determinations and lines were fitted by the programme HYPER.

Expt.	Enzyme Prep.	NaF (mM)	Adenosine (mM)	KmATP (mM)	Vmax[1]	Slope[2]
1	A	0	0	0.162±0.024	260±12	0.622±0.071
			0.1	0.146±0.019	205± 8	0.710±0.073
			0.3	0.110±0.021	149± 9	0.737±0.104
2	A	10	0	0.114±0.016	483±22	0.236±0.026
			0.1	0.106±0.011	394±13	0.377±0.024
			0.3	0.128±0.010	340± 9	0.269±0.023
3	A	10	0	0.222±0.022	846±33	0.262±0.017
			0.1	0.208±0.025	662±30	0.315±0.025
			0.3	0.213±0.030	538±27	0.395±0.040
4	B	0	0	0.072±0.009	208± 6	0.348±0.035
			0.1	0.071±0.016	160± 9	0.442±0.086
			0.3	0.066±0.012	128± 5	0.518±0.076
5	B	0	0	0.078±0.017	190± 9	0.409±0.076
			0.1	0.051±0.010	129± 5	0.391±0.064
			0.3	0.071±0.020	113± 7	0.624±0.151

Table 4: Kinetic constants from programme HYPER: rat brain.
1: pmoles/mg.min; 2: Km/Vmax (mg.min/umole.l).

the simplest further analytical step. The calculated non-competitive inhibition constants Kis and Kii are shown in Table 5 and represent the concentration of adenosine required to double the slope or intercept, respectively, of the double reciprocal plot. If an inhibitor does not modify the Km for the substrate then the two non-competitive inhibition constants are identical. Because of the high standard errors in Kis, it is not obvious from these constants whether adenosine exerts a small but real influence on the Km for ATP or not. Similar non-competitive inhibition by adenosine was observed using adenylate cyclase from rabbit heart (Table 6), which was more sensitive to adenosine inhibition than was brain.

3.2.2. Effect of adenosine analogues on adenylate cyclase.

The effects of fourteen adenosine analogues at 1mM on adenylate cyclase prepared from rabbit intestinal longitudinal muscle by digestion with collagenase are shown in Table 7. The order of potency of analogues which inhibited this cyclase was 6-mercapto- and 2-amino,6-mercapto-purine ribosides > 2'-deoxyadenosine > 3'-deoxyadenosine > adenosine > N^6-hydroxyadenosine > purine riboside. Similar results were obtained using a different

Expt. number	Enzyme prep.	NaF (mM)	KmATP (mM)	Vmax (pmoles/mg.min)	Kis (mM)	Kii (mM)
1	A	0	0.166 ±0.017	260 ± 9	1.75 ±2.17	0.38 ±0.06
2	A	10	0.098 ±0.008	468 ±11	0.37 ±0.10	0.72 ±0.11
3	A	10	0.22 ±0.02	830 ±30	0.55 ±0.19	0.53 ±0.09
4	B	0	0.073 ±0.009	205 ± 6	0.57 ±0.42	0.43 ±0.07
5	B	0	0.073 ±0.013	183 ± 8	0.73 ±0.84	0.38 ±0.07

Table 5: Kinetic constants from programme NONCOMP: rat brain.

Expt.	NaF (mM)	AR[1] (mM)	KmATP (mM)	Vmax[2]	Slope[3]	Kis (mM)	Kii (mM)
		0	0.055 ±0.010	43.3 ± 1.9	1.26 ±0.21		
1	0	0.023	0.065 ±0.019	38.5 ± 2.8	1.69 ±0.43	0.09 ±0.06	0.21 ±0.06
		0.078	0.070 ±0.017	31.3 ± 1.9	2.25 ±0.46		
		0	0.037 ±0.003	131.5 ± 2.7	0.28 ±0.02		
2	10	0.023	0.040 ±0.007	101.3 ± 4.1	0.40 ±0.06	0.07 ±0.02	0.09 ±0.01
		0.078	0.042 ±0.007	71.9 ± 3.0	0.58 ±0.09		

Table 6: Kinetic constants from HYPER and NONCOMP; rabbit heart.
1: adenosine; 2: pmoles/mg.min; 3: Km/Vmax (mg.min/umole.l).

Nucleoside (1mM)	NaF (10mM)	Adenylate cyclase activity (pmoles/mg.min)
-	-	16.5 ± 1.0
-	+	108.5 ± 8.4
Adenosine	+	31.7 ± 4.4 [1] [2]
Adenosine derivatives:		
N^6-hydroxy	+	60.2 ± 7.5 [1] [2]
N^6-methyl	+	95.6 ± 1.6 [2]
N^6-isopentenyl	+	91.4 ± 5.9 [2]
N^6-dimethyl	+	89.8 ± 4.9
2'-deoxy	+	16.5 ± 3.8 [1]
3'-deoxy	+	25.6 ± 1.6 [1]
8-bromo	+	79.3 ± 14.2
Purine riboside derivatives:		
Purine riboside	+	78.5 ± 4.5 [1]
6-methyl	+	97.1 ± 3.2
6-chloro	+	104.0 ± 10.7
6-mercapto	+	13.2 ± 1.6 [1]
6-methylmercapto	+	90.1 ± 6.0
6-methoxy	+	82.5 ± 8.1
2-amino,6-mercapto	+	12.9 ± 2.0 [1]

Table 7: Effect of analogues on longitudinal muscle adenylate cyclase.
Adenylate cyclase activity in the presence of nucleosides and fluoride. Mean and standard error of triplicate incubations. 1: Significantly different from control in the presence of fluoride ($p<0.05$). 2: Relaxes the muscle.

preparation of longitudinal muscle cyclase.

Rat brain adenylate cyclase was inhibited less by 6-mercapto-purine riboside than was longitudinal muscle cyclase and 6-methyl-purine riboside was also inhibitory in that tissue. The order of potency using rat brain was 3'-deoxyadenosine > adenosine > N^6-hydroxyadenosine > 6-methyl-purine riboside > 6-mercapto-purine riboside. The relative potencies 3'-deoxyadenosine > adenosine > N^6-hydroxyadenosine were also found using rat and rabbit heart. 2-Chloroadenosine had little effect on the latter enzyme. The degrees of inhibition induced by adenosine and 3'-deoxyadenosine in rat brain cyclase were not additive (Figure 20), indicating that the two substances probably shared common mechanisms or sites of action.

The influence of theophylline on adenosine inhibition was studied using adenylate cyclase from guinea-pig lung, since this preparation has been reported to be low in phosphodiesterase activity (Weinryb & Michel, 1971). Although this cyclase did contain phosphodiesterase activity (sufficient to hydrolyse 1pmole ^{3}H-cyclic AMP by approximately 90% in 20min), the contamination had no detectable influence on the outcome of adenylate cyclase assays performed in the absence or presence of unlabelled cyclic AMP in the assay medium. Theophylline weakly

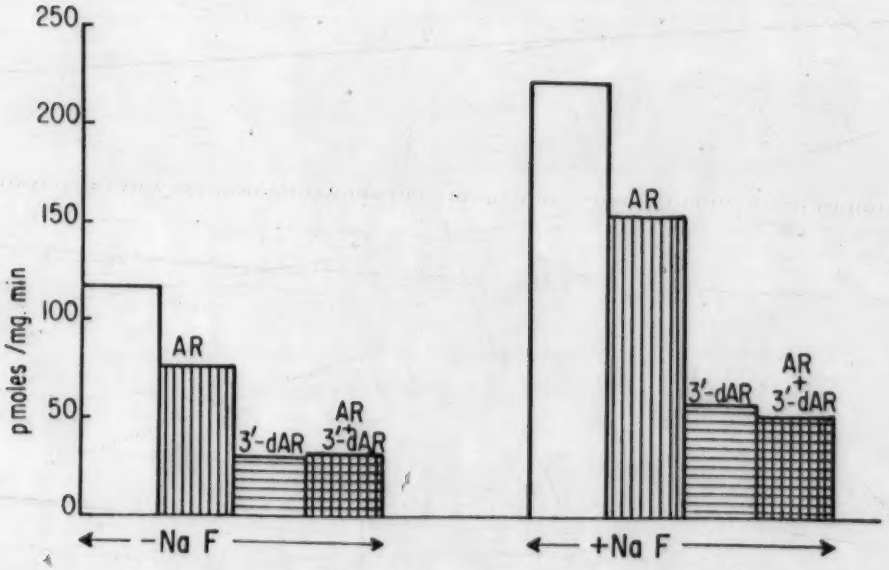


Figure 20: Non-additivity of the effects of adenosine and 3'-deoxyadenosine on cyclase from rat brain.
Means of duplicates. Standard errors are approximately ±1pmole/mg.min.
AR: adenosine 0.3mM; 3'-dAR: 3'-deoxyadenosine 0.64mM.

inhibited this cyclase by up to 35% at 20mM. Higher concentrations were not used because of solubility limits at 4°C. In the presence of 1000uM theophylline, cyclase was inhibited by 14%. The effect of adenosine was, however, unaltered between 1 and 1000uM (Figure 21), indicating that in this system adenosine and theophylline do not act at the same site.

3.2.3. Discussion of adenosine inhibition of adenylate cyclase.

The results presented in this section indicate that the inhibition by adenosine of adenylate cyclase originally reported for rat liver (Moriwaki & Poa, 1970) is not peculiar to that tissue but is also seen with the enzyme from various tissues of several species and thus appears to be a general phenomenon of mammalian systems. Whereas Iwai et al. (1972) found inhibition of protein kinase by adenosine to be competitive with respect to ATP, the kinetics of adenylate cyclase inhibition clearly indicates that adenosine acts non-competitively in this system (Cleland, 1963b).

Although in purified enzyme preparations non-competitive inhibition may be interpreted as interaction

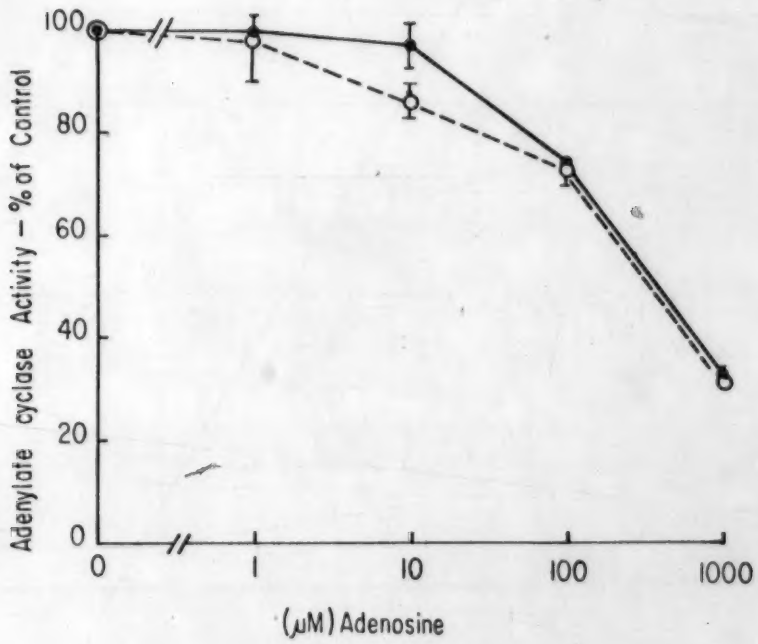


Figure 21: Additivity of the effects of adenosine and theophylline on cyclase from guinea-pig lung.
Means ± standard errors of triplicate determinations. Solid line: theophylline absent. Dashed line: 1mM theophylline present. 100% in the absence of theophylline was 165 ± 2pmoles/mg.min and in its presence 142 ± 4pmoles/mg.min.

of the inhibitor with an allosteric site on the enzyme, this interpretation is unlikely to be precise in the present case since the cyclases studied were components of relatively large membrane fragments. A similar inhibition pattern would be observed under these circumstances if adenosine induced a perturbation in relatively remote parts of the membrane resulting in impaired catalytic activity through distortion of the lipid matrix or protein-protein interactions resulting in a conformational change in the enzyme. Some of the results actually suggest that this may account for at least a portion of the observed inhibition. The greater degree of inhibition due to adenosine with fluoride- or hormone-stimulated as opposed to basal activity (see Table 3) indicates that adenosine effects are not restricted to the catalytic site but may also influence a step common to the stimulatory agents. To the extent that hormone-induced stimulation is dependent on the integrity of the membrane, it is conceivable that distortion of the lipid matrix would influence the effect of stimulatory agents more than basal enzyme activity alone. Alternatively, more than one site of adenosine action must be postulated to account for these observations.

Subsequent to submission of some of these results for publication (McKenzie & Baer, 1973 and Baer & McKenzie,

1973), other reports of inhibition of adenylate cyclase by adenosine have appeared in the literature. Fain et al. (1972) described inhibition of epinephrine activation of cyclase from fat cell ghosts by adenosine and several of its analogues. Their results were in general agreement with those in Table 7, except that the fat cell enzyme resembled that of rat brain in being refractory to 6-mercapto-purine riboside. Fain (1973) later reported that the relative potencies of analogues differed in intact white fat cells as opposed to their ghosts and confirmed the rapidity of onset of adenosine inhibition and its ready reversal with adenosine deaminase. Weinryb & Michel (1974) reported inhibition of guinea-pig lung cyclase by adenosine and several 9-substituted derivatives, some of which were more potent than adenosine. Inhibition of lung cyclase was dependent on magnesium concentration and an analogue, 9-(tetrahydro-2-furyl)adenine, inhibited in an apparently S-linear, I-hyperbolic non-competitive manner. These workers interpreted their observations as indicating negative cooperativity and hence more than one binding site for the analogue under study and for adenosine. While interpretation of a sophisticated analysis as applied to a crude enzyme preparation is difficult as discussed above, the observations of these workers substantiate the assertion that inhibition by adenosine is generally not competitive and that more than one site of

action may be involved. If the magnesium dependence reported by Weinryb & Michel is also general, which is likely since Moriwaki & Foa (1970) reported a similar situation in rat liver, then better estimates of the slope of the double reciprocal plot and of Kis (Tables 4, 5 and 6) might have been obtained in the present study by maintaining a constant Mg:ATP ratio in rate curve experiments. Weinryb & Michel found that this improved the linearity of their double reciprocal plots. Fain (1973) found adenosine inhibition in white fat cells to be independent of calcium concentration.

While the relative potencies of a series of adenosine analogues may vary somewhat between tissues, Table 7 indicates that in longitudinal muscle of the rabbit intestine relaxant activity appears unrelated to inhibition of adenylate cyclase. Not only do pharmacologically inert analogues inhibit this cyclase, but two out of three relaxant analogues fail to do so. The lack of correlation between the two parameters may not therefore be attributed to failure of pharmacologically inactive analogues to gain access to an intramembranal site at which cyclase is inhibited.

In view of the postulated competition between adenosine and theophylline at a stimulatory site on the

adenylate cyclase complex and considering that pharmacological responses to adenosine are frequently antagonised by theophylline, evidence of an interaction between these two substances at the level of adenylate cyclase inhibition was sought. Since inhibition by theophylline of contaminating phosphodiesterase might have been a source of imprecise kinetic measurements, the tissue chosen was guinea-pig lung which proved to be relatively free of phosphodiesterase, as reported by Weinryb & Michel (1971). The lack of effect of an inhibitory concentration of theophylline on adenosine-induced inhibition indicates that the effects of these substances are independent. Since similar results were obtained using rat heart enzyme and 1mM adenosine plus 20mM theophylline (which had no apparent effect on cyclase activity when added alone), the lack of interaction is not peculiar to guinea-pig lung.

Although no stimulation of adenylate cyclase from longitudinal muscle by adenosine or its analogues was observed and thus no support was obtained for the hypothesis that cyclic AMP mediates adenosine responses, a negative result in this case did not refute the hypothesis. The effect of adenosine on adenylate cyclase from rat brain and cerebral cortex was similar to its effect on all other cyclases investigated, although there

is good evidence for the existence of an adenosine-stimulated regulatory unit in this tissue (Sattin & Rall, 1970; Shimizu & Daly, 1970; Huang et al., 1972 and Huang & Daly, 1974). Since labile adenosine-stimulated activity superimposed on the general inhibition has been reported in thrombocytes by Haslam & Lynham (1972), it is possible that the stimulatory activity in brain is also too labile to be observed in broken cell preparations or else that it is obscured by the preponderance of enzyme which is inhibited by adenosine. Similarly, it is conceivable that smooth muscle also contains cyclase-related adenosine receptors which are labile and therefore not detectible in broken cell preparations.

When this work was begun, Davies (1968) had deduced through studies on lipolysis in adipose tissue that adenosine inhibited adenylate cyclase in adipocytes. In 1969 Stock & Westermann reached a similar conclusion with respect to phenylisopropyl-adenosine and suggested that this analogue interfered with ATP binding to adenylate cyclase. Direct evidence of inhibition of cyclase by adenosine had been reported only by Moriwaki & Foa (1970).

In the same year Schaumann et al. (1970) suggested that the cardiovascular effects of adenosine might be due to cyclase inhibition since they were antagonised by the

phosphodiesterase inhibitor theophylline. Later, the coauthors Dietmann & Juhran (1971) retracted this suggestion as applied to coronary vasodilation since antagonism of adenosine effects by theophylline was clearly not functional in that system. This emphasises a major difficulty in ascribing inhibition of cyclase as the mechanism whereby adenosine might induce an effect in any tissue, namely that theophylline antagonises many adenosine responses, such as smooth muscle relaxation and cyclic AMP accumulation in brain slices in what appears to be a non-functional fashion. Since theophylline increases cardiac contractility while adenosine decreases it, it is conceivable that a functional antagonism might exist between these substances on myocardial cyclic AMP levels. This hypothesis gains some support from the results reported above from hearts of various species. Since the dose of adenosine necessary to reduce heart rate is about 100 times that required to increase coronary flow (Schoendorf et al., 1969) it is possible that concentrations sufficiently high (> 10uM) to inhibit cyclase are achieved in the myocardium under these pharmacological conditions. It is also relevant that the metabolic effects of adenosine in perfused rat hearts described by Raberger et al. (1970) as similar to those seen with epinephrine and presumably mediated by cyclic AMP, were observed at a dose of adenosine which did not

reduce heart rate. The available information is therefore not incompatible with the suggestion that cardiac depression, as opposed to coronary vasodilatation, may be induced by adenosine through inhibition of adenylate cyclase.

A physiological role for adenosine in inhibiting lipolysis through inhibition of adenylate cyclase, especially in the presence of hormones, has been proposed by Fain et al. (1972) and Fain (1973), who described inhibition of epinephrine-stimulated cyclic AMP accumulation and adipocyte cyclase by adenosine and several derivatives, as discussed above. Schwabe et al. (1973) detected inhibitory amounts of adenosine in medium containing high concentrations of adipocytes which were no longer responsive to hormone-induced stimulation of cyclase. Adenosine accumulation correlated with decreased cyclase activity over time but not with the extent of previous hormone stimulation or with the rate of lipolysis. While the observations of Schwabe et al. are essential to Fain's suggestion that adenosine may be a physiological feedback inhibitor of adipocyte adenylate cyclase, the lack of correlation between cyclase stimulation by hormones and appearance of adenosine in the medium does not support this hypothesis. Both groups noted a discrepancy between reduction of adipocyte cyclic

AMP levels in the presence of adenosine and the rate of lipolysis. Unexpectedly, perhaps, in view of Davies' (1968) observations that adenosine inhibits also the lipolytic effect of dibutyryl cyclic AMP presumeably by inhibiting the relevant protein kinase, inhibition of lipolysis by adenosine was less pronounced than inhibition of cyclic AMP accumulation. Fain et al. (1972) suggested that not all of the cyclic AMP accumulation due to lipolytic agents is associated with regulation of lipolysis. While adenosine very probably inhibits lipolysis partly through cyclase inhibition at pharmacological concentrations, as has been claimed for phenylisopropyl-adenosine (Stock & Westermann, 1969), it remains unproven that concentrations sufficient to inhibit adenylate cyclase arise under physiological conditions in adipose or any other tissue.

3.3. Tissue cyclic AMP levels and associated relaxant responses.

Cyclic AMP levels in longitudinal muscle strips were estimated as described in Section 2.1.3 and expressed as pmoles per mg protein. Since collagen is insoluble in 0.5N sodium hydroxide at room temperature, protein estimates ignored collagen content, but were roughly proportional to tissue wet weight. The mean protein content of samples was 2.44mg, with standard deviation 0.80 and standard error 0.04mg (n=405).

3.3.1. Variation.

As this work progressed, it became evident that cyclic AMP levels in untreated muscle strips from different animals showed astonishing variation in spite of the fact that all strips behaved similarly mechanically. Consequently, much greater variation in cyclic AMP levels was encountered between different animals within an experimental group than between drug-treated and control strips obtained from one animal and manipulated identically. This fact demanded that statistical analysis of results be performed in a paired or block design whereby drug treated tissues were compared only with controls obtained under identical circumstances. Of 18

animals, each contributing to two complete blocks, the mean control value over both blocks was 6.10pmoles/mg protein, with standard deviation 3.25 and standard error 0.54 (n=36, range 1.77 to 17.75). It should be emphasised that no correlation existed between these values and either protein content of strips or recovery of labelled cyclic AMP. The variation could be observed in the assay of cyclic AMP itself and on subsequent checking of the assay system (see Section 2.1.3.4) high values were invariably confirmed.

The degree of reproducibility of cyclic AMP estimations in fresh strips from the same animal is shown in Table 8. In these experiments strips were under no tension and were equilibrated in aerated Tyrode at 37°C for at least 1h prior to sampling. Group II was sampled 30-40min after group I, after a similar equilibration time. While the mechanical activity of these strips is unknown, strips maintained under tension for similar time periods were spontaneously active. Drug treated strips sampled within 5min of their controls belonged to the population of group I or group II, respectively. The least significant difference between treatments within each group (see Section 3.3.3 and Appendix) was 20-40% of the group control mean. Table 8 emphasises that, even in strips which have been equilibrated as required for

	Group I		Group II	
Expt.	n	cyclic AMP (pmoles/mg protein)	n	cyclic AMP (pmoles/mg protein)
149	6	11.42 ± 2.56	5	4.26 ± 0.32
150	6	11.19 ± 1.40	6	1.91 ± 0.35
151	6	5.04 ± 0.37	6	1.85 ± 0.20

Table 8: **Multiple cyclic AMP measurements from individual animals.**
Mean ± standard error of untreated strips. Groups I and II were frozen by immersion in liquid nitrogen after at least 1h equilibration but separated in time by 30-40min. Treated samples frozen within 5min of controls belonged to the population of their respective group.

optimal spontaneous activity, a close temporal relationship in sampling and identical handling were necessary to ensure that tissue cyclic AMP content was comparable. Cyclic AMP levels were not significantly different between fresh control strips and untreated strips from the same animal which had been stored at 4°C for up to 3h before equilibration at 37°C ($t=0.312$ with 17 degrees of freedom). A similar paired t-test revealed no difference between strips frozen with tongs in the contracted or relaxed state ($t=0.280$ with 17 degrees of freedom).

3.3.2. Sensitivity testing.

In view of the difficulties inherent in estimating drug-induced changes in cyclic AMP levels, samples from each animal were pooled and allocated strictly at random to each treatment group. After the equilibration period, the relaxant response to the treatment was evaluated in duplicate in the appropriate strip and all four strips in each block were frozen within a 10min interval, purified and assayed in the same random order. The statistical significance of the results was calculated by paired t-test or by analysis of variance (see Appendix). The acceptable probability of wrongfully rejecting the null hypothesis was set at 5%. Standard drugs chosen for

comparison with adenosine were isoproterenol, which elevates cyclic AMP levels in smooth muscle, and epinephrine, responses to which more closely resembled adenosine and contained a beta-adrenergic component (Section 3.1.2).

To determine the optimal time interval at which to detect drug-induced changes in cyclic AMP levels and also to estimate the effect on this tissue of high doses of the standard drugs, relaxant responses and tissue cyclic AMP accumulation were estimated in response to 1uM isoproterenol and epinephrine after 10 and 60s of exposure. 1uM Adenosine was included for comparison and results were analysed by paired t-test. As shown in Table 9, adenosine at this concentration induced no change in cyclic AMP levels at either time of exposure, while evoking a mean response of 44% and 23% at 10 and 60s, respectively. Isoproterenol and epinephrine, on the other hand, appreciably elevated cyclic AMP levels at both time intervals. Although epinephrine, like adenosine, induced a rapid initial response, the time course of cyclic AMP accumulation resembled that to isoproterenol, there being greater accumulation after 60 than after 10s. The increase in response to epinephrine after 10s just failed in significance. In view of these results, tissues were subsequently sampled for cyclic AMP estimation after 60s

Drug(1uM)		10 seconds	60 seconds
Isoproterenol	% Response[1]	52.3 ± 6.2	85.0 ± 3.7
	cAMP[2]	+1.93 ± 0.82	+6.30 ± 2.52
	p	<0.05	<0.05
Epinephrine	% Response[1]	86.2 ± 3.9	87.8 ± 2.1
	cAMP[2]	+0.83 ± 0.40	+1.91 ± 0.26
	p	0.1>p>0.05	<0.05
Adenosine	% Response[1]	43.8 ± 7.1	22.8 ± 2.9
	cAMP[2]	+0.86 ± 1.16	+0.30 ± 0.36
	p	n.s.	n.s.

Table 9: Relaxation and change in cyclic AMP levels in response to 1uM isoproterenol, epinephrine and adenosine. Paired comparisons, n=6. 1: relaxant response; 2: change in cyclic AMP content (pmoles/mg protein).

of exposure to drugs.

As reported in Section 3.1, after 10min of exposure, 0.1mM theophylline had been found to antagonise responses to adenosine while potentiating those to the standard drugs. Since no other purine antagonist had been revealed among the series of adenosine analogues and since theophylline antagonises cyclic AMP accumulation in brain slices and thrombocytes in response to adenosine, it was of interest to determine the influence of theophylline on adenosine-induced changes in cyclic AMP content of this tissue. If theophylline were indeed a competitive antagonist of adenosine responses, it would antagonise any causative increase in cyclic AMP levels. To serve as controls, the effect of theophylline was also evaluated on cyclic AMP accumulation in response to isoproterenol and epinephrine, whose responses it potentiated.

The statistical method of evaluating potentiation or antagonism between two drugs on any parameter is by means of a Model I 2-way analysis of variance, with each drug representing a factor which may be present or absent. Each block therefore consisted of a control tissue, a tissue treated with adenosine or one of the standard drugs, a theophylline-treated tissue and a tissue exposed to both theophylline and the test drug, all tissues being

from the same animal. The exposure time to theophylline was 11min and to other drugs 60s. Six replications from different animals comprised the complete block. In this case the statistical term 'interaction', which denotes non-additivity of the effects of each factor when samples are exposed to both factors together, is synonymous with drug interaction, i.e. potentiation or antagonism. If statistically significant interaction does not exist, this analysis has the advantage of harbouring 'hidden replicates' whereby the main effect of each drug is assessed both in the absence and presence of the other drug. A further discussion of the statistics employed is contained in the Appendix.

To determine the lower limit of detectibility of changes in cyclic AMP content in response to drugs, the lowest dose of isoproterenol reported in the literature to elevate smooth muscle cyclic nucleotide levels (0.05uM) was employed. The effect of theophylline on this response was concomitantly evaluated as described above. The data are shown in 3-dimensional array in Table 10. The mean relaxant responses in the drug-treated strips were: isoproterenol 43.3 ± 5.6%, theophylline +4.7 ± 6.2% and isoproterenol in the presence of theophylline 72.0 ± 3.7%, where a positive value represents increased amplitude of spontaneous contractions. The true potentiatory effect of

	EXPT	Cyclic AMP (pmoles/mg protein)	
		-ISOPROTERENOL	+ISOPROTERENOL
-THEOPHYLLINE	141	7.18	5.95
	142	5.06	7.14
	143	8.66	8.88
	144	3.48	4.00
	145	2.50	2.91
	146	3.91	4.75
+THEOPHYLLINE	141	7.27	9.98
	142	5.62	8.87
	143	11.13	9.36
	144	5.43	4.84
	145	2.39	3.33
	146	3.44	5.12

Table 10: Tissue cyclic AMP levels 60s after 0.05uM isoproterenol ± 0.1mM theophylline.
Individual estimates of cyclic AMP content of tissues from six animals.

theophylline was evaluated on the strips receiving combined treatment and was 43.3 ± 7.8%. The outcome of the analysis of variance is shown in Table 11. A significant additional variance component when compared with the error variance (error MS) was contributed by different animals and by treatment with 0.1mM theophylline, which increased cyclic AMP levels by 1.03 ± 0.37 pmoles/mg protein. The effect of 0.05uM isoproterenol (+0.76 ± 0.44 pmoles/mg protein) approached significance, while combined treatment with isoproterenol plus theophylline induced a change in cyclic AMP levels which was not significantly different from the sum of the individual effects of theophylline and isoproterenol. Thus, theophylline potentiated relaxant responses to this dose of isoproterenol while producing only an additive increment in the cyclic AMP content of strips exposed to both drugs.

When the mixed adrenergic agonist epinephrine was similarly investigated, 0.05uM induced a relaxant response of 42.8 ± 7.2% in strips exposed to this drug alone and 55.2 ± 5.8% in strips pretreated with 0.1mM theophylline for 10min. The response of the muscle to theophylline alone was +11.3 ± 5.3% and potentiation by theophylline of epinephrine responses within individual muscle strips was 28.2 ± 6.7%. The cyclic AMP content of strips from six

Source of variation	df	SS	MS	Fs	P
Animals	5	123.54	24.71	22.9	p<0.001*
0.05uM Isoproterenol	1	3.42	3.42	3.30	0.1>p>0.05
0.1mM Theophylline	1	6.37	6.37	6.15	0.05>p>0.025*
Interaction	1	0.48	0.48	0.47	ns
Error	15	15.50	1.03		
Total	23	149.30			

Table 11: Analysis of variance table: 0.05uM isoproterenol versus 0.1mM theophylline.
Abbreviations:- df: degrees of freedom; SS: sum of squares; MS: mean square (SS/df); Fs: value of F-distribution (MS/error MS); p: probability of F occurring by chance; *: significant at 5%; ns: p>25%.
Relaxant responses:- isoproterenol alone 43.3 ± 5.6%; isoproterenol in the presence of theophylline 72.0 ± 3.7%. Theophylline increased the amplitude of spontaneous contractions by 4.7 ± 6.2% and potentiated isoproterenol responses by 43.3 ± 7.8%.

animals and the results of the analysis of variance are shown in Tables 12 and 13 respectively. In this analysis neither 0.05uM epinephrine nor 0.1mM theophylline significantly elevated cyclic AMP levels although the content of strips varied significantly between animals. Once again no additional variance component could be attributed to interaction between these drugs on this biochemical parameter.

In summary, the following results were obtained, despite the variability discussed in Section 3.3.1, in experiments designed to test the sensitivity of the assay system to drug-induced changes in cyclic AMP content.

1. 1uM Isoproterenol, which relaxed the muscle by 85 ± 3.7% at 60s, significantly elevated the cyclic AMP content of the tissue after either 10 or 60s of exposure. Accumulation was greater at 60 than at 10s (Table 9). 0.05uM Isoproterenol, the smallest concentration reported in the literature to influence smooth muscle cyclic AMP, caused 43.3 ± 5.6% relaxation in this tissue and an increase in cyclic AMP content of 0.76 ± 0.44 pmole/mg protein at 60s, which just failed to be significant at the prechosen level of 5%.
2. 0.05uM Epinephrine, a mixed adrenergic agonist, failed to elevate cyclic AMP levels significantly at 60s, while causing 42.8 ± 7.2% relaxation of the muscle. 1uM

	EXPT	Cyclic AMP (pmoles/mg protein)	
		-EPINEPHRINE	+EPINEPHRINE
-THEOPHYLLINE	128	6.39	6.68
	131	8.34	7.55
	132	10.18	5.97
	134	3.46	2.72
	136	2.54	5.06
	137	1.77	3.29
+THEOPHYLLINE	128	9.21	10.13
	131	7.49	6.53
	132	6.57	7.33
	134	4.62	2.75
	136	4.66	2.97
	137	2.74	3.49

Table 12: Tissue cyclic AMP levels 60s after 0.05uM epinephrine ± 0.1mM theophylline.
Individual estimates of cyclic AMP content of tissues from six animals.

Source of variation	df	SS	MS	Fs	P
Animals	5	117.60	23.52	11.87	p<0.001 *
0.05uM Epinephrine	1	0.46	0.46	0.232	ns
0.1mM Theophylline	1	0.92	0.92	0.464	ns
Interaction	1	0.03	0.03	0.015	ns
Error	15	29.70	1.98		
Total	23	148.71			

Table 13: Analysis of variance table: 0.05uM epinephrine versus 0.1mM theophylline.
Abbreviations as in Table 11.
Relaxant responses:- epinephrine alone 42.8 ± 7.2%; epinephrine in the presence of theophylline 55.2 ± 5.8%. Theophylline increased the amplitude of spontaneous contractions by 11.3 ± 5.3% and potentiated epinephrine responses by 28.2 ± 6.7%.

Epinephrine, producing 87.8 ± 2.1% relaxation, significantly increased cyclic AMP content after 60 but not after 10s of exposure (Table 9).

3. Although 0.1mM theophylline, which did not decrease the amplitude of spontaneous activity, potentiated responses to 0.05uM isoproterenol or epinephrine by 43.3 ± 7.8% and 28.2 ± 6.7%, respectively, no significant interaction on the parameter of cyclic AMP content of the tissue was detected between theophylline and either standard drug (Tables 11 and 13).

3.3.3. Effect of adenosine on cyclic AMP levels.

As reported in Table 9, 1uM adenosine did not influence cyclic AMP content while relaxing the tissue by 22.8 ± 2.9% at 60s. Cyclic AMP estimations in the absence and presence of 10uM adenosine and 0.1mM theophylline are shown in Table 14 and results of the analysis in Table 15. Neither 10uM adenosine nor theophylline significantly modified this parameter, nor was there an interaction between the drugs. The mean relaxant response to this dose of adenosine was 31.0 ± 5.5%, which corresponded to 45.8 ± 5.7% of the maximal tissue response to 400uM adenosine. In the presence of theophylline, the response to this dose of adenosine was reduced to 10.5 ± 1.9%, or 15.5 ± 2.1% of maximal adenosine-induced relaxation. In

	EXPT	Cyclic AMP (pmoles/mg protein)	
		-ADENOSINE	+ADENOSINE
-THEOPHYLLINE	128	6.45	8.84
	131	7.93	7.79
	132	17.75	5.90
	133	10.50	5.89
	134	3.71	4.22
	136	4.43	4.38
+THEOPHYLLINE	128	8.13	9.41
	131	6.67	8.08
	132	6.95	4.01
	133	6.74	8.49
	134	5.33	5.59
	136	4.10	4.98

Table 14: Tissue cyclic AMP levels 60s after 10uM adenosine ± 0.1mM theophylline.
Individual estimates of cyclic AMP content of tissues from six animals.

Source of variation	df	SS	MS	Fs	P
Animals	5	67.92	13.58	1.752	0.25>p>0.1
10uM Adenosine	1	5.14	5.14	0.663	ns
0.1mM Theophylline	1	3.61	3.61	0.466	ns
Interaction	1	11.19	11.19	1.442	0.25>p>0.1
Error	15	116.32	7.75		
Total	23	204.19			

Table 15: Analysis of variance table: 10uM adenosine versus 0.1mM theophylline.
Abbreviations as in Table 11.
Relaxant responses:- adenosine alone 31.0 ± 5.5%; adenosine in the presence of theophylline 10.5 ± 1.9%. Theophylline increased the amplitude of spontaneous contractions by 8.0 ± 5.3% and antagonised adenosine responses by 68.3 ± 4.9%.

strips exposed to both adenosine and theophylline, the degree of antagonism exerted by theophylline was calculated as 68.3 ± 4.9%.

Since 0.1mM theophylline had been chosen as a dose which did not relax the muscle and hence which minimally inhibited phosphodiesterase as indicated in Tables 11, 13 and 15, the effect of a phosphodiesterase inhibitory concentration of methylisobutylxanthine, which did not antagonise adenosine responses, was determined on relaxant responses to a high dose of 100uM adenosine and on cyclic AMP accumulation in the tissue. Results are shown in Tables 16 and 17. Methylisobutylxanthine was present for 10min before addition of adenosine and relaxed the muscle by 44.5 ± 5.5%. 100uM Adenosine induced relaxant responses of 66.2 ± 6.7% (89.7 ± 4.2% of adenosine maximum) in the absence of methylisobutylxanthine and 76.3 ± 4.0% (106.7 ± 6.3% maximal) in its presence. Potentiation of adenosine responses by methylisobutylxanthine was calculated in strips exposed to both as 14.7 ± 5.5%. The analysis of variance revealed that 10uM methylisobutylxanthine significantly elevated cyclic AMP levels, the mean difference being 1.42 ± 0.58 pmoles/mg protein. At this dose of adenosine, which relaxed the tissue more than did methylisobutylxanthine, the change in cyclic AMP content was +0.80 ± 0.56 and was not

	EXPT	Cyclic AMP (pmoles/mg protein)	
		-ADENOSINE	+ADENOSINE
-MIX[1]	141	3.99	5.69
	142	6.90	5.03
	143	2.98	4.56
	144	2.94	3.26
	145	2.26	3.21
	146	2.27	2.52
+MIX[1]	141	7.24	6.71
	142	5.59	11.50
	143	5.02	4.28
	144	4.18	4.54
	145	3.11	3.03
	146	2.82	4.60

Table 16: Tissue cyclic AMP levels 60s after 100uM adenosine ± 10uM methylisobutylxanthine.
Individual estimates of cyclic AMP content of tissues from six animals. 1: methylisobutylxanthine.

Source of variation	df	SS	ms	Fs	p
Animals	5	59.58	11.92	7.64	p<0.001 *
100uM Adenosine	1	3.86	3.86	2.48	0.25>p>0.1
10uM MIX[1]	1	12.06	12.06	7.74	0.025>p>0.01*
Interaction	1	0.59	0.59	0.378	ns
Error	15	23.41	1.56		
Total	23	99.50			

Table 17: Analysis of variance table: 100uM adenosine versus 10uM methylisobutylxanthine.
Abbreviations as in Table 11; 1: methylisobutylxanthine. Relaxant responses:- adenosine alone 66.2 ± 6.7%; adenosine in the presence of MIX 76.3 ± 4.0%. MIX reduced the amplitude of spontaneous contractions by 44.5 ± 5.5% and potentiated adenosine responses by 14.7 ± 5.5%.

significant. No interaction was detected.

Since 100uM adenosine produced a response equivalent to 90% of the maximal tissue response to adenosine, it was obvious that any influence of higher doses on cyclic AMP levels could not be correlated to muscle relaxant activity. Nevertheless, it was of interest to determine whether adenosine or ATP, like epinephrine, could induce cyclic AMP accumulation at high doses. Since it was no longer necessary to evaluate relaxant responses, six strips from one animal were exposed simultaneously to drug treatment to yield an estimate of the error of each observation. The three treatment groups consisted of control, adenosine or ATP (100uM) to re-evaluate the outcome of the previous experimental group and adenosine or ATP (1mM). The effect of drug treatment on tissue cyclic AMP content was evaluated for each experiment by means of a one-way analysis of variance (see Appendix). The simplest technique for comparing group means is through the computation of the least significant difference. This is derived by substituting the error mean square (calculated by analysis of variance) as the closest estimate of population variance in the equation for t (see Appendix) and represents the difference between means which would yield a t-value equal to that at the prechosen significance level, in this case 5%. The

results of the individual analyses are expressed in Table 18. The difference in control values between parts (a) and (b) in the same experiment are described in Section 3.3.1 and discussed later. If it is accepted that adenosine and ATP influence cyclic AMP levels identically, the results expressed in Table 18 may be pooled and analysed by two-way analysis of variance to increase the sensitivity of the procedure to small changes in tissue cyclic AMP. Since in this case one of the main factors (animals) is random, the interaction term reflects error introduced by different outcomes in each of the individual one-way analyses (Table 18). To account for this, the significance of the difference between treatment groups was tested by dividing the treatment mean square by the interaction mean square to yield the F-value of the treatments. As shown in Table 19, interaction produced a significant error and no significant difference was detected among the treatment groups, including control. There was thus no indication that adenosine or ATP enhanced tissue cyclic AMP content even at 1mM concentrations. These results were as anticipated from inspection of Table 18.

Expt.	Treatment	cyclic AMP[1]	Difference from control	L.S.I.[2]	L.S.I.[2] % Control
	Control	11.19 ± 1.40			
150a	0.1mM AR[3]	8.01 ± 0.32	-3.18	+2.32	20.7
	1mM AR[3]	8.92 ± 0.81	-2.27		
	Control	1.91 ± 0.35			
150b	0.1mM ATP	2.20 ± 0.30	+0.29	+0.78	40.8
	1mM ATP	2.29 ± 0.25	+0.33		
	Control	5.04 ± 0.37			
151a	0.1mM AR[3]	5.27 ± 0.38	+0.23	+1.04	20.6
	1mM AR[3]	5.20 ± 0.53	+0.16		
	Control	1.85 ± 0.20			
151b	0.1mM ATP	2.03 ± 0.15	+0.18	+0.48	25.9
	1mM ATP	1.95 ± 0.23	+0.10		

Table 18: One-way analysis of variance with six tissues per group.
Cyclic AMP content is expressed as mean ± s.e. of six observations. None of the four complete analyses indicated significant drug effects. 1: pmoles/mg protein; 2: least significant increase, calculated as described in the Appendix and expressed in the last column as percent of control mean; 3: adenosine.

Source of variation	df	SS	MS	Fs	p
Subgroups	11	684.94	62.27		
Treatments	2	4.76	2.38	0.509	ns
Animals	3	652.18	217.39	117.82	p<0.001*
Interaction	6	28.01	4.67	2.53	0.05>p>0.025*
Error	60	110.17	1.845		
Total	71	795.65			

Table 19: Analysis of variance table: adenosine or ATP at 0.1 and 1mM versus animals.
The data summarised in Table 18 were pooled and analysed in a block design. *: Significant at 5%; ns: p>25%.

3.3.4. Discussion of cyclic AMP measurements.

Variability of control measurements of cyclic AMP between different experiments is tacitly reported by all authors performing such measurements in smooth muscle, but is seldom commented upon. Inspection of published work reveals inherent variability of the same order as reported here, no matter the units of measurement employed, e.g. Bueding et al. (1966): 0.09-0.48 nanomoles per g wet weight (guinea-pig taenia coli); Polacek & Daniel (1971): 55.3 ± 16.8 (mean ± s.e., n=6) ng/100mg (rat uterus), implying a standard deviation of about 33 units and 95% confidence limits of 0-120 units; Andersson (1972): 82 ± 18 (mean ± s.e., n=6-8) nmoles/g (rabbit colon), implying 95% confidence limits of about 0-170 units and Inatomf et al. (1974): 0.55 ± 0.07 (mean ± s.e., n=6) pmoles/mg wet weight (guinea-pig taenia caeci) or 95% confidence limits of about 27-83 units. The latter workers remarked that since greater variability was observed between different batches of guinea-pigs, only one batch was used per experimental set. The extraordinary variability is often advertantly or inadvertantly concealed within a relatively small standard error term which refers properly to the estimate of the mean value but does not accurately describe the population of individual controls. The use of mean values for control or treated groups has therefore

been eschewed in the presentation of results in this section and numerical differences only are expressed as mean ± standard error.

Table 8 indicates that differences such as those observed between animals may be reproduced within one animal under certain circumstances. Since the data in this table were obtained from strips which were not under tension when sampled and whose mechanical activity was therefore unknown, it is not possible to extrapolate these observations to working strips in satisfactory mechanical condition. Whereas cyclic AMP content appeared to diminish over a relatively short time in strips under no imposed tension, no significant difference was detected between control levels of fresh strips and of those stored for up to 3h at 4°C before mounting under tension. Similarly, no correlation was apparent between total time at 37°C and cyclic AMP content in either fresh or stored spontaneously active strips. Since sampling order, along with treatment group, was assigned strictly randomly throughout, any effect of time on cyclic AMP content would not influence the outcome of the analyses of variance reported above.

Despite strict experimental protocol, considerable intra-animal variation was observed in spontaneously

active strips (Tables 10, 12, 14 and 16). Such tables of experimental results are rarely published in the literature, but Bueding et al. (1966) illustrate cyclic AMP increments in response to 0.025uM epinephrine as +17 to +156% and to 0.5uM epinephrine as -4 to +94%. Similar variation in drug-induced changes is obvious in other work by calculation of the 95% confidence limits of the population of differences and may reflect either variable sensitivity of different animals to a particular treatment or intra-animal variation of cyclic AMP levels or both.

In this muscle, no significant difference was detected between samples frozen in the contracted or relaxed state. It remains possible, however, that transient changes in cyclic AMP content might be revealed during the contraction/relaxation cycle if strips could reliably be frozen at precise points in the oscillations. This would require extensive study at relatively low temperatures since at 37°C the frequency of spontaneous contractions (17/min) does not allow the necessary precision. If fluctuations in cyclic AMP are responsible for spontaneous activity in this tissue, as suggested by Bowman & Hall (1970), a time lapse might be expected between peak cyclic AMP levels and minimal tension developement, depending on the time necessary for the tissue to respond. Diamond & Hartle (1974) have, however,

found no change in either cylic AMP or cyclic GMP at four points in the time course of spontaneous contractions in rat uterus. As procedural protocol in the present experiments, control and drug-treated tissues were frozen as close as possible to the peak of spontaneously induced tension.

In view of the wide range of control and treated values in this and other types of smooth muscle, evaluation of drug-induced changes in cyclic AMP content presents conceptual difficulties, firstly, because such changes are invariably within the range of controls and, secondly, because it is necessary to decide whether drug-induced changes are a function of their respective control level or are independent events. Results have been presented by different authors in different ways, e.g. as arithmetic increments by Polacek & Daniel (1971) and Andersson (1972) and as a function of control by Bueding et al. (1966) and Inatomi et al. (1974). The last mentioned workers perpetrated a statistical error which appears attractive if increments are expressed as a function of control, by performing a paired t-test on data expressed as percentage of control. Since the t-test is a form of analysis of variance and the control group expressed as 100% contained no variance at all, the test performed was invalid. The purpose of its use was

undoubtedly to boost the effects of the three lowest of four doses of isoproterenol into apparent significance. Bueding et al. (1966) do not indicate their method of statistical analysis.

Statistical procedures for detection of changes which are a function of control depend upon the use of the logarithms of the data obtained. The logarithmic transformation is also indicated in two other circumstances:

1) where the logarithms of the data better satisfy the assumptions of analysis of variance than do the data themselves and

2) where unwanted interaction between treatments threatens to obscure the outcome of the analysis, logarithmic transformation may reduce the interaction effect to insignificance.

Calculations with the logarithms of the data reported above revealed that the transformation did not appreciably reduce the error term in the analyses of variance. Since no statistical advantage was gained by this manipulation and since its use was likely to obscure any real interaction between drug effects, the logarithmic transformation was statistically undesireable. The final decision of how to interpret the data therefore rested on whether or not drug effects are properly expressed as

functions of control. Since control levels varied widely in strips which appeared mechanically equivalent, a large proportion of tissue cyclic AMP content must be located in cells other than smooth muscle or in subcellular compartments unrelated to mechanical activity, assuming that the pathway proposed by Andersson (1972) for cyclic AMP-induced relaxation exists. Since there is no a priori reason to assume communication between such compartments and likewise no a posteriori evidence from analysis of the data, results have been presented above as if drug-induced increments were independent of basal cyclic AMP content.

Sensitivity testing revealed this system to be as responsive to drugs as are other types of smooth muscle. Isoproterenol at 0.05uM marginally elevated cyclic AMP by 0.76 ± 0.44pmoles/mg protein while relaxing the muscle by 43%. Methylisobutylxanthine at 10uM induced a similar relaxant response and significantly enhanced cyclic AMP content by 1.42 ± 0.58pmoles/mg protein. Nevertheless, adenosine at 1-100uM, which relaxed the muscle by up to 66%, did not significantly influence tissue cyclic AMP levels. Since epinephrine, a mixed adrenergic agonist which evoked relaxant responses resembling those to adenosine elevated cyclic AMP at high but not at lower doses, a supramaximal dose of adenosine was also employed to determine whether adenosine resembled epinephrine in

this respect. Since primary drug-induced effects, as opposed to secondary consequences of the response, should be observable in muscle under no imposed tension, the effects of 100uM and 1mM adenosine and ATP were evaluated in groups of six strips suspended together in Tyrode, rather than mounted in an organ bath. Whereas a 20% elevation (+0.48 to +2.32pmoles/mg protein, see Table 18) would have been significant, the effects of these concentrations of adenosine and ATP were neither significant nor dose-dependent.

The fact that the least significant difference in these one-way analyses appears to be a function of control probably reflects proportionately increasing experimental error with measurement of larger cyclic AMP values. This is inevitable since the reciprocal of recovery of labelled tracer, a source of error which is independent of total cyclic AMP, is multiplied by cyclic AMP (as measured in the assay) to yield the estimate of total cyclic AMP.

Although the accepted criteria for ascribing the mechanism of action of a drug to mediation by cyclic AMP demand that the drug elevate total tissue cyclic AMP levels, this may be an overly simplistic view. Since nucleotides are known to be compartmentalised within cells, it is conceivable that a smooth muscle relaxant

might elevate cyclic AMP only in a compartment associated with relaxation.

Adenosine has been shown to inhibit mammalian adenylate cyclase quite nonspecifically, even in tissues where an adenosine-sensitive regulatory subunit is believed to exist. It remains conceivable that both actions on cyclase may exist concomitantly in one tissue and that cyclase inhibition in one cellular compartment may obscure the effects of stimulation of sensitive cyclase in a different functional compartment. If this were the case in smooth muscle, theophylline, which is known to be an effective antagonist at the stimulatory but apparently not at the inhibitory site, and which also antagonised adenosine-induced relaxation in this tissue, might reveal a net decrease in cyclic AMP levels in response to adenosine. Since this possible effect of theophylline was not observed, there remains no evidence of an adenosine-sensitive adenylate cyclase in this tissue. Since accumulation of cyclic AMP through stimulation of adenylate cyclase should be enhanced when phosphodiesterase is inhibited, 10uM methylisobutyl-xanthine should have revealed any stimulatory effect of 100uM adenosine on cyclase. Since no interaction between these drugs on cyclic AMP levels was detected, one must again conclude that adenosine does not stimulate cyclic

AMP formation. Methylisobutylxanthine potentiated responses to 100uM adenosine by 15%, but owing to the marked decrease in amplitude of spontaneous contractions induced by the phosphodiesterase inhibitor, this effect appears artifactual.

It was hoped that interaction between drugs on the parameter of cyclic AMP accumulation would be demonstrated using theophylline and isoproterenol or epinephrine. For the reason just discussed, the optimal dose of theophylline for observing potentiation was one which did not relax the muscle. Since phosphodiesterase inhibition has been associated with smooth muscle relaxation (Kukovetz & Poech, 1970, Lugnier et al., 1972 and Andersson, 1973b) this dose of theophylline was anticipated to exhibit only threshold inhibition of phosphodiesterase. Since in two out of three experiments theophylline failed to elevate cyclic AMP levels significantly, this was obviously true. The failure to detect significant interaction in these analyses may reflect the fact that response is not linearly related to cyclic AMP accumulation. If, for example, the dose-effect curve between change in cyclic AMP and relaxation is sigmoidal, even an undetectably small increment in cyclic AMP, caused by a dose of theophylline which alone did not relax the muscle, might promote a considerably greater

response to another agonist because of additive effects on cyclic AMP. Alternatively, interaction may be present but so small at these doses relative to the inherent error as to be insignificant. In all analyses of variance where interaction between drugs was sought, the ratio of responses to adenosine or catecholamines in the individual strips in which cyclic AMP was measured, accurately reflected the true ratio of potentiation. This ratio of potentiation was determined as the ratio of responses before and after treatment with theophylline or methylisobutylxanthine in strips exposed to both drugs.

Apart from the lack of stimulatory effects of adenosine on cyclic AMP levels, it is also noteworthy that no significant decrease in cyclic AMP content was observed using up to 1mM adenosine. This effect might have been based on the marked (up to 71%) inhibition of isolated adenylate cyclase from this tissue. At high concentrations adenosine permeates biological membranes by simple diffusion (see Section 1.1.1) and 1mM extracellular ^{14}C-adenosine appears capable of enhancing intracellular concentrations of ^{14}C-label in erythrocytes to about 10uM within one minute (Roos & Pfleger, 1972). These workers did not, however, identify the chemical nature of the intracellular label. Since a battery of enzymes with different Km values for adenosine exists in all tissues,

it is certain that a considerable proportion of the label had been converted to adenine nucleotides, inosine, hypoxanthine or other metabolites. Even if similar rapid entry of adenosine occurs in smooth muscle at an extracellular concentration of 1mM, it is unlikely that an intracellular concentration of 10uM of adenosine per se would be maintained. Adenosine has been found to diminish cyclic AMP levels in adipocytes and fat cell ghosts (Fain et al., 1972, Fain, 1973 and Schwabe et al., 1973), but only in the presence of lipolytic agents. It has also been reported that AMP and ADP inhibit cyclase while inosine does not (Moriwaki & Foa, 1970). The degree of inhibition of cyclase in any tissue exposed to high concentrations of adenosine may therefore also depend upon the particular metabolic products formed in that tissue. Failure of 1mM adenosine to decrease cyclic AMP levels in smooth muscle may therefore be due to its inability to raise the concentration of adenosine or nucleotides at the site of inhibition of adenylate cyclase to 10uM, the minimum for observable cyclase inhibition in broken cells. This finding constitutes some support for the idea that adenosine inhibition involves an intracellular site, most likely the catalytic unit of the enzyme system facing the cell interior.

3.4. Conclusions.

The inhibition of the amplitude of spontaneous contractions induced by adenosine and several phosphorylated and non-phosphorylated analogues over the dose range of 0.1-100uM is concluded to be due to a direct action on the muscle and to be mediated by an extracellular receptor. The moieties found to be necessary for receptor activation by purine nucleosides were a primary or secondary amino group on the N^6-position and hydroxyl groups at the 2'- and 3'-positions. Adenosine and its nucleotides were equipotent and 8-bromoadenosine was inactive. Responses to adenosine and nucleotides were antagonised by prior exposure to effective doses of these agents, possibly by means of an intracellular action of adenosine. This effect was readily reversible after washing. Investigation of a series of available adenosine analogues did not reveal a competitive antagonist. Antagonism was, however, exerted by 0.1mM theophylline at which concentration it minimally modified the amplitude of spontaneous contractions but potentiated responses to epinephrine and isoproterenol. The antagonism could be overcome by high concentrations of agonist. Since adenosine responses were not appreciably modified by the phosphodiesterase inhibitors papaverine and methylisobutylxanthine or by imidazole, no

pharmacological evidence of cyclic AMP mediation in responses was obtained and it is unlikely that theophylline antagonism was related to its ability to inhibit phosphodiesterase.

Adenylate cyclase, prepared from various tissues of several species, was uniformly inhibited by adenosine at or above 10uM. In basal and fluoride-stimulated enzyme from rat brain and rabbit heart, inhibition was found to be noncompetitive with respect to ATP. In no tissue was significant stimulation of broken cell adenylate cyclase observed with adenosine. Analogues sharing with adenosine the capacity to inhibit adenylate cyclase from rabbit intestinal longitudinal muscle bore no correlation with analogues which relaxed the muscle. Although these findings do not relate directly to the proposed hypothesis of adenosine action, they indicate that the ubiquitous and pronounced inhibition of cyclase seen with adenosine is not necessarily related to pharmacological responses, although such a possibility seems to exist in adipose tissue.

Whereas isoproterenol, epinephrine and the phosphodiesterase inhibitor 1-methyl,3-isobutylxanthine all significantly enhanced cyclic AMP content of rabbit intestinal longitudinal muscle strips, adenosine did not

significantly influence cyclic AMP levels over a dose range of 1-1000uM. ATP was also ineffective at 100 and 1000uM. Since neither the adenosine antagonist theophylline not the phosphodiesterase inhibitor methylisobutylxanthine modified this outcome, no support was obtained for the hypothesis that adenosine- and ATP-induced inhibition of spontaneous activity in this muscle involves mediation by cyclic AMP.

Since total tissue cyclic AMP appears to bear no correlation with muscle tone, the hypothesis might be further tested using the technique for measuring newly-formed cyclic AMP by prelabelling specific nucleotide pools (Shimizu et al., 1969). This technique measures more directly changes in cyclic AMP production due to drug-induced stimulation of adenylate cyclase. Such experiments would be particularly worthwhile if, pursuant to the finding of competitive antagonists, adenosine were found to have more than one mechanism of action in smooth muscle. It is further possible that, rather than elevating tissue cyclic AMP, adenosine may relocate the nucleotide within the cell by inducing changes in the cytoskeleton. The investigation of the latter possibility is unfortunately as yet beyond the scope of current technological prowess. In view of the results of this study, however, which failed to detect significant

elevation of cyclic AMP even at high doses, it is more likely that an explanation to the problem of how these substances relax smooth muscle will be revealed by studying their effects on calcium binding in subcellular membrane fractions, particularly plasma membrane.

Since autoinhibition of alpha-adrenergic responses in vascular and uterine smooth muscle has been associated with the existence of subthreshold, opposing beta-adrenergic effects of epinephrine, the interesting possibility exists that the autoinhibition by adenosine and ATP is also a manifestation of opposing effects of these substances in intestinal muscle. Further investigation of this phenomenon might reveal whether transport into the tissue is a prerequisite of this effect and whether a structure-activity relationship exists for the ability of analogues to induce autoinhibition. In such a way, a further understanding of the complexity of the adenosine response in smooth muscle might be obtained.

BIBLIOGRAPHY

Afonso, S. (1970): Inhibition of coronary vasodilating action of dipyridamole and adenosine by aminophylline in the dog. Circ. Res. 26:743-752.

Afonso, S., T.J. Ansfield, T.B. Berndt & G.G. Rowe (1972): Coronary vasodilator responses to hypoxia before and after aminophylline. J. Physiol. 221:589-599.

Afonso, S. & G.S. O'Brien (1970): Inhibition of cardiovascular metabolic and hemodynamic effects of adenosine by aminophylline. Am. J. Physiol. 219:1672-1674.

Afonso, S. & G.S. O'Brien (1971): Mechanism of enhancement of adenosine action by dipyridamole and lidoflazine, in dogs. Arch. Int. Pharmacodyn. Ther. 194:184-196.

Afonso, S, G.S. O'Brien & C.W. Crumpton (1968): Enhancement of coronary vasodilating action of ATP and adenosine by lidoflazine. Circ. Res. 22:43-48.

Andersson, R. (1972): Role of cyclic AMP and Ca^{++} in the metabolic and relaxing effects of catecholamines in intestinal muscle. Acta Physiol. Scand. 85:312-322.

Andersson, R. (1973a): Role of cyclic AMP and Ca^{++} in mechanical and metabolic events in isometrically contracting vascular smooth muscle. Acta Physiol. Scand. 87:84-95.

Andersson, R. (1973b): Cyclic AMP as a mediator of the relaxing action of papaverine, nitroglycerine, diazoxide and hydralazine in intestinal and vascular smooth muscle. Acta Pharmacol. Toxicol. 32:321-336.

Andersson, R. (1973c): Relationship between cyclic AMP, phosphodiesterase activity, calcium and contraction in intestinal smooth muscle. Acta Physiol. Scand. 87:348-358.

Andersson, R. & K. Nilsson (1972): Cyclic AMP and calcium in relaxation in intestinal smooth muscle. Nature (New Biol.) 238:119-120.

Appleman, M.M., L. Birnbaumer & H.N. Torres (1966): Factors affecting the activity of muscle glycogen synthetase. III: The reaction with adenosine triphosphate, Mg^{++}, and cyclic 3',5'-adenosine

monophosphate. Arch. Biochem. Biophys. 116:39-43.

Axelsson, J. & B. Holmberg (1969): The effects of extracellularly applied ATP and related compounds on electrical and mechanical activity of the smooth muscle taenia coli from the guinea-pig. Acta Physiol. Scand. 75:149-156.

Baer, H-P. (1974): Cyclic nucleotides and smooth muscle. Adv. Cyclic Nucleotide Res. 4:195-237.

Baer, H.-P. (1975): Measurement of adenyl cyclase and cyclic AMP in smooth muscle. in Methods in Pharmacology III. Smooth muscle:596-611, E.E. Daniel & D.M. Paton, eds., Appleton Press.

Baer, H.-P., G.I. Drummond & J. Gillis (1968): Studies on the specificity and mechanism of action of adenosine deaminase. Arch. Biochem. Biophys. 123:172-178.

Baer, H.-P. & O. Hechter (1969): Adenyl cyclase and hormone action, I. Effects of adrenocorticotropic hormone, glucagon and epinephrine on the plasma membrane of rat fat cells. Proc. Natl. Acad. Sci. 63:350-356.

Baer, H.-P. & J.F. Henderson (1972): Properties of adenyl cyclase from Ehrlich ascites cells. Can. J. Biochem. 50:1003-1009.

Baer, H.-P. & S.G. McKenzie (1973): On the action of adenosine upon cardiac and other adenyl cyclases. in Recent Advances in Studies on Cardiac Structure and Metabolism, Vol. 3, Myocardial Metabolism:311-318, N.S. Dhalla, ed., University Park Press.

Baer, H.-P. & L.P. Simonson (1975): Inhibition of extracellular and purified 5'-nucleotidase from rat heart. in Recent Advances in Studies on Cardiac Structure and Metabolism, Vol. 2, N.S. Dhalla, ed., University Park Press, (in press).

Barnett, A., D.D. Greenhouse & R.I. Taber (1968): A new type of drug enhancement: increased maximum response to cumulative noradrenaline in the isolated rat vas deferens. Br. J. Pharmacol. 33:171-176.

Bellemann, P. & H. Scholz (1975): Dissociation of theophylline uptake and inotropic effect in myocardial tissue: influence of temperature, pH and calcium. Br. J. Pharmacol. 54:75-81.

Bell, C. (1974): Effects of dipyridamole on neurogenic and non-neurogenic dilator responses of arterial smooth muscle. Br. J. Pharmacol. 52:13-17.

Berne, R.M. (1963): Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. Am. J. Physiol. 204:317-322.

Berne, R.M., R. Rubio, J.D. Dobson & R.R. Cornish (1971): Adenosine and adenine nucleotides as possible mediators of cardiac and skeletal muscle blood flow regulation. Circ. Res. 28 [Suppl. I]:I115-I119.

Bishop, H., A.C. Frazer & R. Schneider (1963): The nature of the antiperistaltic factor from wheat gluten. Br. J. Pharmacol. 21:238-243.

Bittar, N. & T.J. Pauly (1970): Potentiation by dipyridamole of myocardial reactive hyperemia in the dog. Pharmacol. Res. Commun. 2:231-242.

Bittar, N. & T.J. Pauly (1971): Role of adenosine in reactive hyperemia of the dog heart. Experientia 27 :153-154.

Bluse, A.J., C. Dalton & H. Sheppard (1973): Adenosine-mediated elevation of cyclic 3':5'- adenosine monophosphate concentrations in cultured mouse neuroblastoma cells. Proc. Natl. Acad. Sci. 70:3099-3102.

Born, G.V.R., R.J. Haslam & M. Goldman (1965): Comparative effectiveness of adenosine analogues as inhibitors of blood-platelet aggregation and as vasodilators in man. Nature 205:678-680.

Born, G.V.R., A.J. Honour & J.R.A. Mitchell (1964): Inhibition by adenosine and by 2-chloroadenosine of the formation and embolization of platelet thrombi. Nature 202:761-765.

Bowman, W.C. & M.T. Hall (1970): Inhibition of rabbit intestine mediated by alpha- and beta-adrenoceptors. Br. J. Pharmacol. 38:399-415.

Bretschneider, H.J., A. Frank, U. Bernard, K. Kochsiek & F. Scheler (1959): Die Wirkung eines Pyrimido-pyrimidin-Derivates auf die Sauerstoffversorgung des Herzmuskels. Arzneim. Forsch. 9:49-59.

Bueding, E., E. Bulbring, G. Gercken, J.T. Hawkins & H.

Kuriyama (1967): The effect of adrenaline on the adenosine triphosphate and creatine phosphate content of intestinal smooth muscle. J. Physiol. 193:187-212.

Bueding, E., R.W. Butcher, J. Hawkins, A.R. Timms & E.W. Sutherland (1966): Effect of epinephrine on cyclic adenosine 3',5'-phosphate and hexose phosphates in intestinal smooth muscle. Biochim. Biophys. Acta 115 :173-178.

Burger, R.M. & J.M. Lowenstein (1970): Preparation and properties of 5'-nucleotidase from smooth muscle of small intestine. J. Biol. Chem. 245:6274-6280.

Burnstock, G. (1972): Purinergic nerves. Pharmacol. Revs. 24:509-581.

Burnstock, G. G. Campbell, D. Satchell & A. Smythe (1970): Evidence that ATP of a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves of the gut. Br. J. Pharmacol. 40 :668-688.

Burnstock, G., T. Cocks, B. Paddle & J. Staszewska-Barczak (1975): Evidence that prostaglandin is responsible for the 'rebound contraction' following stimulation of non-adrenergic, non-cholinergic ('purinergic') inhibitory nerves. Eur. J. Pharmacol. 31:360-362.

Burnstock, G., B. Dumsday & A. Smythe (1972a): Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. Br. J. Pharmacol. 44:451-461.

Burnstock, G., D.G. Satchell & A. Smythe (1972b): A comparison of the excitatory and inhibitory effects of non-adrenergic, non-cholinergic nerve stimulation and exogenously applied ATP on a variety of smooth muscle preparations from different vertebrate species. Br. J. Pharmacol. 46:234-242.

Chiba, S., K. Kubota & K. Hashimoto (1973): Inhibition of the negative chronotropic action of adenosine by caffeine in the dog. Eur. J. Pharmacol. 21:281-285.

Clark, R.B., R. Gross, Y.-F. Su & J.P. Perkins (1974): Regulation of adenosine 3':5'-monophosphate content in human astrocytoma cells by adenosine and the adenine nucleotides. J. Biol. Chem. 249:5296-5303.

Cleland, W.W. (1963a): Computer programmes for processing

enzyme kinetic data. Nature 198:463-465.

Cleland, W.W. (1963b): The kinetics of enzyme-catalysed reactions with two or more substrates or products. II. Inhibition: nomenclature and theory. Biochim. Biophys. Acta 67:173-196.

Coleman, R.A. & G.P. Levy (1974): A non-adrenergic inhibitory nervous pathway in guinea-pig trachea. Br. J. Pharmacol. 52:167-174.

Daniel, E.E. & J. Crankshaw (1974): Relation of c-AMP to relaxation of pulmonary artery. Blood Vessels 11:295-311.

Daniel, E.E. & J. Irwin (1965): On the mechanism whereby certain nucleotides produce contractions of smooth muscle. Can. J. Physiol. Pharmacol. 43:89-109.

Davies, J.I. (1968): In vitro regulation of the lipolysis of adipose tissue. Nature 218:349-352.

Deuticke, B. & E. Gerlach (1966): Kompetitive Hemmung der Adenosin-Desaminase als moegliche Ursache der coronardilatierenden Wirkung einer Pyrimidopyrimidin-Verbindung. Naunyn-Schmiedebergs Arch. Pharmacol. 255:107-119.

Diamond, J. & D.K. Hartle (1974): Cyclic nucleotide levels during spontaneous uterine contractions. Can. J. Physiol. Pharmacol. 52:763-767.

Dietmann, K. & W. Juhran (1971): Korrelation zwischen den Kreislaufwirkung und der Hemmung der Lipolyse bei einen neunen Adenosin-Derivat. Naunyn-Schmiedebergs Arch. Pharmacol. 269:467.

Dobson, J.G., R. Rubio & R.M. Berne (1971): Role of adenine nucleotides, adenosine and inorganic phosphate in the regulation of skeletal muscle blood flow. Circ. Res. 29:375-384.

Dole, V.P. (1961): Effect of nucleic acid metabolites on lipolysis in adipose tissue. J. Biol. Chem. 236:3125-3130.

Dole, V.P. (1962): Insulin-like actions of ribonucleic acid, adenylic acid, and adenosine. J. Biol. Chem. 237:2758-2762.

Drury, A.N. (1936): The physiological activity of nucleic

acid and its derivatives. Physiol. Rev. 16:292-325.

Drury, A.N. & A. Szent-Gyorgyi (1929): The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. J. Physiol. 68:213-237.

Dumsday, B. (1971): Atropine-resistance of the urinary bladder. J. Pharm. Pharmacol. 23:222-225.

Ebadi, M.S. (1972): Firefly luminescence in the assay of cyclic AMP. Adv. Cyclic Nucleotide Res. 2:89-108.

Einstein, R., J.A. Angus, L.B. Cobbin & M.H. Maguire (1972): Separation of vasodilator and negative chronotropic actions in analogues of adenosine. Eur. J. Pharmacol. 19:246-250.

Fain, J.N. (1973): Inhibition of adenosine cyclic 3',5'-monophosphate accumulation in fat cells by adenosine, N⁶-(phenylisopropyl)adenosine, and related compounds. Mol. Pharmacol. 9:595-604.

Fain, J.N., R.H. Pointer & W.F. Ward (1972): Effects of adenosine nucleosides on adenylate cyclase, phosphodiesterase, cyclic adenosine monophosphate accumulation, and lipolysis in fat cells. J. Biol. Chem. 247:6866-6872.

Falk, G. (1956): Nonspecificity of ATP-contraction of living muscle. Science 123:632-633.

Feldberg, W. & C. Hebb (1948): The stimulating action of phosphate compounds on the perfused superior cervical ganglion of the cat. J. Physiol. 107:210-221.

Gerlach, E., B. Deuticke & R.H. Dreisbach (1963): Der Nucleotid-Abbau im Herzmuskel bei Sauerstoffmangel und seine moegliche Bedeutung fur die Coronardurchblutung. Naturwissenschaften 50:228-229.

Gilman, A.G. (1970): A protein binding assay for adenosine 3':5'-cyclic monophosphate. Proc. Natl. Acad. Sci. 67 :305-312.

Grossman, A. & R.F. Furchgott (1964): The effects of various drugs on calcium exchange in the isolated guinea pig left auricle. J. Pharmacol. Exp. Ther. 145 :162-172.

de Gubareff, T. & W. Sleator (1965): Effects of caffeine

on mammalian atrial muscle, and its interaction with adenosine and calcium. J. Pharmacol. Exp. Ther. 148 :202-214.

Guimaraes, S. (1972): The slopes of cumulative and non-cumulative dose-response curves for noradrenaline and isoprenaline. Eur. J. Pharmacol. 17:44-49.

Gulyassy, P.F. (1971): Inhibition of cyclic 3',5'-nucleotide phosphodiesterase by adenine compounds. Life Sci. 10:451-461.

Guthrie, J.R. & W.G. Nayler (1967): Interaction between caffeine and adenosine on calcium exchangeability in mammalian atria. Arch. Int. Pharmacodyn. Ther. 170 :249.

Hampton, A., P. Harper & T. Sasaki (1972): Substrate properties of cycloadenosines with adenosine aminohydrolase as evidence for the conformation of enzyme-bound adenosine. Biochemistry 11:4736-4739.

Haslam, R.J. & J.A. Lynham (1972): Activation and inhibition of blood platelet adenyl cyclase by adenosine or by 2-chloroadenosine. Life Sci. (II) 11 :1143-1154.

Hoffmann, P.C. & G.T. Okita (1965): Penetration of ATP into the myocardium. Proc. Soc. Exp. Biol. Med. 119 :573-576.

Hopkins, S.V. (1973a): The action of ATP in the guinea-pig heart. Biochem. Pharmacol. 22:335-340.

Hopkins, S.V. (1973b): The potentiation of the action of adenosine on the guinea-pig heart. Biochem. Pharmacol. 22:341-348.

Hopkins, S.V. & R.G. Goldie (1971): A species difference in the uptake of adenosine by heart. Biochem. Pharmacol. 20:3359-3365.

Huang, M. & J.W. Daly (1974): Adenosine-elicited accumulation of cyclic AMP in brain slices. Potentiation by agents which inhibit uptake of adenosine. Life Sci. 14:489-503.

Huang, M., H. Shimizu & J.W. Daly (1972): Accumulation of cyclic adenosine monophosphate in incubated slices of brain tissue. 2. Effects of depolarising agents, membrane stabilisers, phosphodiesterase inhibitors

and adenosine analogues. J. Med. Chem. 15:462-466.

Huang, Y.-C. & R.G. Kemp (1971): Properties of a phosphodiesterase with high affinity for adenosine 3',5'-cyclic phosphate. Biochemistry 10:2278-2283.

Hulme, M.E. & A.H. Weston (1974a): Some effects of dipyridamole, hexobendine and lidoflazine on inhibitory processes in rabbit duodenum. Br. J. Pharmacol. 50:609-611.

Hulme, M.E. & A.H. Weston (1974b): The accumulation of adenosine in rabbit intestinal muscle. Br. J. Pharmacol. 50:569-574.

Imai, S., A.L. Riley & R.M. Berne (1964): Effect of ischemia on adenine nucleotides in cardiac and skeletal muscle. Circ. Res. 15:443-450.

Inatomi, N., I. Takayanagi, M. Uchida & K. Takagi (1974): Intracellular cyclic AMP level and intestinal smooth muscle relaxation. Eur. J. Pharmacol. 26:73-76.

Iso, T. (1973): Effects of cyclic 3',5'-AMP and related compounds on catecholamine-induced contraction of isolated guinea-pig vas deferens. Jap. J. Pharmacol. 23:717-721.

Iwai, H., M. Inamasu & S. Takeyama (1972): Inhibition of the protein kinase by adenine compounds: competitive inhibition with respect to ATP. Biochem. Biophys. Res. Commun. 46:824-830.

James, T.N. (1965): The chronotropic action of ATP and related compounds studied by direct perfusion of the sinus node. J. Pharmacol. Exp. Ther. 149:233-247.

Juhran, W. & K. Dietmann (1970): Zur Regelung der Coronardurchblutung im akuten Sauerstoffmangel. Pflügers Arch. 315:105-109.

Kalsner, S. (1974): A vasodilator innervation to the central artery of the rabbit ear. Br. J. Pharmacol. 52:5-12.

Katori, M. & R.M. Berne (1966): Release of adenosine from anoxic hearts. Circ. Res. 19:420-425.

Kim, T.S., J. Shulman & R.A. Levine (1968): Relaxant effect of cyclic adenosine 3',5'-monophosphate on the isolated rabbit ileum. J. Pharmacol. Exp. Ther. 163

:36-42.

Kolassa, N., K. Pfleger & M. Traem (1971): Species differences in action and elimination of adenosine after dipyridamole and hexobendine. Eur. J. Pharmacol. 13:320-325.

Kolassa, N., M. Traem & K. Pfleger (1970): Speciesunterschiede bei der Beeinflussung der Wirkung und Elimination von Adenosin durch Dipyridamol und Hexobendin. Naunyn-Schmiedebergs Arch. Pharmacol. 266 :373.

Kraupp, O. (1969): Zum Wirkungsmechanismus der Coronardilatatoren. Naunyn-Schmiedebergs Arch. Pharmacol. 263:144-155.

Kuchii, M., J.T. Miyahara & S. Shibata (1973a): [^{3}H]-adenine nucleotide and [^{3}H]-noradrenaline release evoked by electrical field stimulation, perivascular nerve stimulation and nicotine from the taenia of the guinea-pig caecum. Br. J. Pharmacol. 49:258-267.

Kuchii, M., J.T. Miyahara & S. Shibata (1973b): [^{3}H]-adenosine nucleotide and [^{3}H]-noradrenaline uptake by cold stored guinea pig taenia caecum; mechanical effects and release of [^{3}H]-adenosine nucleotide by noradrenaline, papaverine and nitroglycerine. Br. J. Pharmacol. 49:642-650.

Kuchii, M., J.T. Miyahara & S. Shibata (1974): Effect of 6-hydroxydopamine on [^{3}H]-adenine nucleotide and [^{3}H]-noradrenaline release from the guinea-pig taenia caecum evoked by electrical field stimulation, nicotine and perivascular nerve stimulation. Br. J. Pharmacol. 51:577-583.

Kuebler, W. & H.J. Bretschneider (1963): Die Permeation von Adenosin durch die Erythrocytenmembran des Hundes. Pfluegers Arch. 277:141-149.

Kuebler, W. & H.J. Bretschneider (1964): Kompetitive Hemmung der katalysierten Adenosindiffusion als Mechanismus der coronarerweiternden Wirkung eines Pyrimido-pyrimidin-Derivates. Pfluegers Arch. 280 :141-157.

Kuebler, W., P.G. Spiekermann & H.J. Bretschneider (1970): Influence of dipyridamole (Persantin) on myocardial adenosine metabolism. J. Mol. Cell. Cardiol. 1:23-38.

Kukovetz, W.R. & G. Poech (1970): Inhibition of cyclic 3',5'-nucleotide phosphodiesterase as a possible mode of action of papaverine and similarly acting drugs. Naunyn-Schmiedebergs Arch. Pharmacol. 267:189-194.

Kuo, J.F., B.K. Krueger, J.R. Sanes & P. Greengard (1970): Cyclic nucleotide-dependent protein kinases V. Preparation and properties of adenosine 3',5'-monophosphate-dependent protein kinase from various bovine tissues. Biochim. Biophys. Acta 212:79-91.

Leslie, S.W., J.L. Borowitz & T.S. Miya (1973): Adenosine analogs: structure-activity relationships in vascular and intestinal smooth muscle. J. Pharm. Sci. 62:1449-1452.

Liu, J., C.L. Prosser & D.D. Job (1969): Ionic dependence of slow waves and spikes in intestinal muscle. Am. J. Physiol. 217:1542-1547.

Lowry, O.H., N.J. Rosenbrough, A.L. Farr & R.J. Randal (1951): Protein measurement with folin phenol reagent. J. Biol. Chem. 193:265-275.

Lugnier, C., Y. Bertrand & J.C. Stoclet (1972): Cyclic nucleotide phosphodiesterase inhibition and vascular smooth muscle relaxation. Eur. J. Pharmacol. 19:134-136.

Lundholm, L. (1957): The mechanism of the vasodilator effect of adrenaline. 1. Effect on skeletal muscle blood vessels. Acta Physiol. Scand. 39 [Suppl. 133]:3-52.

McDougal, M.R. & J.L. Borowitz (1972): The effect of adenosine and cold storage on the response of the guinea-pig ileum to various agents. Arch. Int. Pharmacodyn. Ther. 195:177-184.

McKenzie, S.G. & H.-P. Baer (1973): On the mechanism of adenyl cyclase inhibition by adenosine. Can. J. Physiol. Pharmacol. 51:190-196.

McNeill, J.H., M. Nassar & T.M. Brody (1969): The effect of theophylline on amine-induced cardiac phosphorylase activation and cardiac contractility. J. Pharmacol. Exp. Ther. 165:234-241.

Madinaveitia, J. & J. Raventos (1949): Antimalarial compounds as antagonists of adeosine. Br. J. Pharmacol. 4:81-92.

Mihich, E., D.A. Clark & F.S. Philips (1954): Effect of adenosine analogs on isolated intestine and uterus. J. Pharmacol. Exp. Ther. 111:335-342.

Mills, D.C.B. & J.B. Smith (1971): The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3',5'-cyclic monophosphate in platelets. Biochem. J. 121:185-196.

Miura, M., S. Tominaga & K. Hashimoto (1967): Potentiation of reactive hyperemia in the coronary and femoral circulation by the selective use of 2,6-bis-(diethanolamino)-4,8-dipiperidino-pyrimido[5,4-d] pyrimidine. Arzneim. Forsch. 17:976-979.

Miyamoto, E., J.F. Kuo & P. Greengard (1969): Cyclic nucleotide-dependent protein kinases. III. Purification and properties of adenosine 3',5'-monophosphate-dependent protein kinase from bovine brain. J. Biol. Chem. 244:6395-6402.

Moir, T.W. & T.D. Downs (1972): Myocardial reactive hyperemia: comparative effects of adenosine, ATP, ADP, and AMP. Am. J. Physiol. 222:1386-1390.

Moriwaki, K. & P.O. Poa (1970): Inhibition of rat liver adenyl cyclase by adenosine and adenine nucleotides. Experientia 26:22.

Nair, K.G. (1966): Purification and properties of 3',5'-cyclic nucleotide phosphodiesterase from dog heart. Biochemistry 5:150-157.

Nakanishi, H. & H. Takeda (1972): The possibility that adenosine triphosphate is an excitatory transmitter in guinea-pig seminal vesicle. Jap. J. Pharmacol. 22 :269-270.

Nayler, W.G. (1963): Effect of caffeine on cardiac contractile activity and radiocalcium movement. Am. J. Physiol. 204:969-974-974.

Nayler, W.G. & J.R. Hasker (1966): Effect of caffeine on calcium in subcellular fractions of cardiac muscle. Am. J. Physiol. 211:950-954.

Nesheim, B.-I., J.-B. Osnes & I. Oye (1975): Role of cyclic adenosine 3',5'-monophosphate in the isoprenaline-induced relaxation of the oestrogen dominated rabbit uterus. Br. J. Pharmacol. 53:403-407.

Oliver, J.M. & A.R.P. Paterson (1971): Nucleoside transport: I. A mediated process in human erythrocytes. Can. J. Biochem. 49:262-270.

Olsson, R.A. (1970): Changes in content of purine nucleosides in canine myocardium during coronary occlusion. Circ. Res. 26:301-306.

Olsson, R.A., M.K. Gentry & J.A. Snow (1973): Steric requirements for binding of adenosine to a membrane carrier in canine heart. Biochim. Biophys. Acta 311 :242-250.

Olsson, R.A., J.A. Snow, M.K. Gentry & G.P. Frick (1972): Adenosine uptake by canine heart. Circ. Res. 31:767-778.

Onabanjo, A.O. & B.G. Maegraith (1970): The probable pathogenic role of adenosine in malaria. Br. J. Exp. Pathol. 51:581-585.

Palmer, G.C. (1971): Characteristics of the hormonal induced cyclic adenosine 3',5'-monophosphate response in the rat and guinea-pig lung in vitro. Biochim. Biophys. Acta 252:561-566.

Paterson, A.R.P. & J.M. Oliver (1970): Nucleoside transport. II. Inhibition by p-nitrobenzylthioguanosine and related compounds. Can. J. Biochem. 49 :271-274.

Paul, B., M.F. Chen & A.R.P. Paterson (1975): Inhibitors of nucleoside transport. A structure-activity study using human erythrocytes. J. Med. Chem. (in press).

Peck, W.A., J. Carpenter & K. Messinger (1974): Cyclic 3',5'-adenosine monophosphate in isolated bone cells. II. Responses to adenosine and parathyroid hormone. Endocrinology 94:148-154.

Pfleger, K., D. Niederau & I. Volkmer (1969a): Ein Beitrag zum Wirkungsmechanismus von Dipyridamol: Hemmung der Adenosinaufnahme in Erythrocyten durch Dipyridamol. Naunyn-Schmiedebergs Arch. Pharmacol. 265:118-130.

Pfleger, K. & H. Schoendorf (1969): Zur Aufklaerung des Wirkungsmechanismus von Dipyridamol. Arzneim. Forsch. 19:97-98.

Pfleger, K., E. Seifen & H. Schoendorf (1969b): Potenzierung der Adenosinwirkung am Herzen durch

Inosin. Biochem. Pharmacol. 18:43-51.

Plagemann, P.G.W. (1971): Nucleoside transport by Novikoff rat hepatoma cells growing in suspension culture: Specificity and mechanism of transport reactions and relationship to nucleoside incorporation into nucleic acids. Biochim. Biophys. Acta 233:688-701.

Plagemann, P.G.W. & J.R. Sheppard (1974): Competitive inhibition of the transport of nucleosides, hypoxanthine, choline and deoxyglucose by theophylline, papaverine and prostaglandins. Biochem. Biophys. Res. Commun. 56:869-875.

Polacek, I., J. Bolan & E.E. Daniel (1971): Accumulation of adenosine 3',5'-monophosphate and relaxation in the rat uterus. Can. J. Physiol. Pharmacol. 49:999-1004.

Polacek, I. & E.E. Daniel (1971): Effect of alpha- and beta- adrenergic stimulation on the uterine motility and adenosine 3',5'-monophosphate level. Can. J. Physiol. Pharmacol. 49:988-998.

Raberger, G., O. Kraupp, W. Strulinger, G. Nell & J.J. Chirikdjian (1970): The effects of intracoronary infusion of adenosine on cardiac performance, blood supply and on myocardial metabolism in dogs. Pflügers Arch. 317:20-34.

Raberger, G., M. Weissel & O. Kraupp (1971): The dependence of the effects of i.cor. administered adenosine and of coronary conductance on the arterial pH, pCO2 and buffer capacity in dogs. Naunyn-Schmiedebergs Arch. Pharmacol. 271:301-310.

Raberger, G., M. Weissel, O. Kraupp & J.J. Chirikdjian (1973a): Circulatory and metabolic effects of adenosine in the hind limb of intact dogs. Naunyn-Schmiedebergs Arch. Pharmacol. 277:227-237.

Raberger, G., M. Weissel & O. Kraupp (1973b): Wirkung von Hexobendin auf die koronare Wirksamkeit einer generalisierten Hypoxie sowie i. cor. und i.v. Einzelinjektionen von Adenosin an Hunden. Arzneim. Forsch. 23:764-768.

Rand, M.J. & A. Stafford (1957): The influence of oubain on the response of the guinea-pig heart to acetylcholine, adenosine and vagal stimulation. Arch. Int. Pharmacodyn. Ther. 99:3-4.

Rand, M., A. Stafford & R.H. Thorp (1955): The potentiation of the action of adenosine on the guinea-pig heart by ouabain. J. Pharmacol. Exp. Ther. 114:119-125.

Richman, H.G. & L. Wyborny (1964): Adenine nucleotide degradation in the rabbit heart. Am. J. Physiol. 207 :1139-1145.

Rikimaru, A., Y. Fukushi & T. Suzuki (1971): Effects of imidazole and phentolamine on the relaxant responses of guinea-pig taenia coli to transmural stimulation and to adenosine triphosphate. Tohoku J. Exp. Med. 105:199-200.

Rodbell, M. (1964): Metabolism of isolated fat cells. I: Effects of hormones on glucose metabolism and lipolysis. J. Biol. Chem. 239:375-380.

Rodbell, M. (1967): Metabolism of isolated fat cells. V: Preparation of 'ghosts' and their properties; adenyl cyclase and other enzymes. J. Biol. Chem. 242:5744-5750.

Roos, H. & K. Pfleger (1972): Kinetics of adenosine uptake by erythrocytes, and the influence of dipyridamole. Mol. Pharmacol. 8:417-425.

Rubio, R., R.M. Berne & M. Katori (1969): Release of adenosine in reactive hyperaemia of the dog heart. Am. J. Physiol. 216:56-62.

Satchell, D.G., A. Lynch, P.M. Bourke & G. Burnstock (1972): Potentiation of the effects of exogenously applied ATP and purinergic nerve stimulation on the guinea-pig taenia coli by dipyridamole and hexobendine. Eur. J. Pharmacol. 19:343-350.

Sattin, A. & T.W. Rall (1970): The effect of adenosine and adenine nucleotides on cyclic adenosine 3',5'-phosphate content of guinea-pig cerebral cortex slices. Mol. Pharmacol. 6:13-23.

Schaumann, von W., W. Juhran & K. Dietman (1970): Antagonismus der Kreislaufwirkungen von Adenosin durch Theophyllin. Arzneim. Forsch. 20:372-377.

Schoendorf, H., W. Russel & K. Pfleger (1969): Dosis-Wirkungs-Beziehungen zwischen Coronardurchfluss und Herzfrequenz fur Adenosin. Experientia 25:44-45.

Schrader, J., R.M. Berne & R. Rubio (1972): Uptake and metabolism of adenosine by human erythrocyte ghosts. Am. J. Physiol. 223: 159-166.

Schulz, J. & B. Hamprecht (1973): Adenosine 3',5'-monophosphate in cultured neuroblastoma cells: effect of adenosine, phosphodiesterase inhibitors and benzazepines. Naunyn-Schmiedebergs Arch. Pharmacol. 278: 215-225.

Schwabe, U., R. Ebert & H.C. Erbler (1973): Adenosine release from isolated fat cells and its significance for the effects of hormones on cyclic 3',5'-AMP levels and lipolysis. Naunyn-Schmiedebergs Arch. Pharmacol. 276: 133-148.

Shimizu, H. & J. Daly (1970): Formation of cyclic adenosine 3',5'-monophosphate from adenosine in brainslices. Biochim. Biophys. Acta 222: 465-473.

Shimizu, H., J.W. Daly & C.R. Creveling (1969): A radioisotopic method for measuring the formation of adenosine 3',5'-cyclic monophosphate in incubated slices of brain. J. Neurochem. 16: 1609-1619.

Simon, L.N., R.J. Bauer, R.L. Tolman & R.K. Robins (1970): Calf intestine adenosine deaminase. Substrate specificity. Biochemistry 9: 573-577.

Somlyo, A.P., A.V. Somlyo & V. Smiesko (1972): Cyclic AMP and vascular smooth muscle. Adv. Cyclic Nucleotide Res. 1: 175-194.

Somlyo, A.P. & C. Woo (1967): Beta-adrenergic auto-inhibition of the effect of noradrenaline on avian pulmonary artery. J. Pharm. Pharmacol. 19: 59-61.

Somlyo, A.V., G. Haeusler & A.P. Somlyo (1970): Cyclic adenosine monophosphate: potassium-dependent action on vascular smooth muscle membrane potential. Science 169: 490-491.

Somlyo, A.V. & A.P. Somlyo (1968): Electromechanical and pharmacomechanical coupling in vascular smooth muscle. J. Pharmacol. Exp. Ther. 159: 129-145.

Spedding, M., A.J. Sweetman & D.F. Weetman (1975): Antagonism of adenosine 5'-triphosphate-induced relaxation by 2-2'-pyridylisatogen in the taenia of the guinea-pig caecum. Br. J. Pharmacol. 53: 575-583.

Stafford, A. (1966): Potentiation of adenosine and the adenine nucleotides by dipyridamole. Br. J. Pharmacol. 28:218-227.

Stock, K. & E. Westermann (1969): On the mechanism of the anti-lipolytic effect of phenylisopropyl-adenosine (PIA) in vitro. Naunyn-Schmiedebergs Arch. Pharmacol. 264:311-312.

Su, C., J.A. Bevan & G. Burnstock (1971): ^{3}H-adenosine triphosphate: release during stimulation of enteric nerves. Science 173:336-338.

Ther, L., R. Muschaweck & J. Hergott (1957): Antagonismus zwischen Adenosin und Methyl-Xanthinen am Reitz-leitungssystem des Herzens. Naunyn-Schmiedebergs Arch. Pharmacol. 231:586-590.

Tominaga, S., T. Suzuki & T. Nakamura (1973): Evaluation of roles of potassium, inorganic phosphate, osmolarity, pH, pCO2, pO2, and adenosine or AMP in exercise and reactive hyperemias in canine hindlimb muscles. Tohoku J. Exp. Med. 109:347-363.

Tomita, T. & H. Watanabe (1973): Factors controlling myogenic activity in smooth muscle. Philos. Trans. R. Soc. Lond. 265:73-85.

Tomiyama, A., I. Takayanagi & K. Takagi (1973): Relaxation of intestinal smooth muscle and calcium movements. J. Pharm. Pharmacol. 25:65-68.

Triner, L., Y. Vulliemoz, M. Verosky & G.G. Nahos (1970): The effect of catecholamines on adenyl cyclase activity in rat uterus. Life Sci. 9:707-712.

Wadsworth, R.M. (1972): The effects of aminophylline on the increased myocardial blood flow produced by systemic hypoxia or by coronary artery occlusion. Eur. J. Pharmacol. 20:130-132.

Wedd, A.M. (1931): The action of adenosine and certain related compounds on the coronary flow of the perfused heart of the rabbit. J. Pharmacol. Exp. Ther. 41:355-366.

Weinryb, I. & I.M. Michel (1971): Divergent effects of theophylline on adenylate cyclase preparations from guinea-pig heart and lung. Experientia 27:1386.

Weinryb, I. & I.M. Michel (1974): Potent magnesium-

dependent inhibition of adenylate cyclase from guinea-pig lung by adenosine and other 9-substituted adenines. Biochim. Biophys. Acta 334:218-225.

Weston, A.H. (1971): Inhibition of the longitudinal muscle of rabbit duodenum. Br. J. Pharmacol. 43:428P-429P.

Weston, A.H. (1973a): The effect of desensitization to adenosine triphosphate on the peristaltic reflex in guinea-pig ileum. Br. J. Pharmacol. 47:606-608.

Weston, A.H. (1973b): Nerve-mediated inhibition of mechanical activity in rabbit duodenum and the effects of desensitization to adenosine and several of its derivatives. Br. J. Pharmacol. 48:302-308.

Williams, A.M. & G.A. le Page (1958): Purine metabolism in mouse ascites tumor cells. II. In vitro incorporation of preformed purines into nucleotides and polynucleotides. Cancer Res. 18:554-561.

Wollenberger, A., E.-G. Krause & G. Heier (1969): Stimulation of 3',5'-cyclic AMP formation in dog myocardium following arrest of blood flow. Biochem. Biophys. Res. Commun. 36:664-670.

Woo, Y.-T., J.F. Manery & E.E. Dryden (1974): Theophylline inhibition of nucleoside transport and its relation to cyclic AMP in skeletal muscle. Can. J. Physiol. Pharmacol. 52:1063-1073.

APPENDIX.

The equation for a Model I one-way analysis of variance is represented as:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where Y_{ij} is the measured value of the jth sample in the ith group

μ is the grand mean of the population

α_i is the fixed deviation of the mean of group i from μ, due to a particular treatment

ε_{ij} is an independent, normally distributed variable, with a mean of 0 and variance equal to the population variance, σ^2.

The expected error mean square (within groups) is σ^2 and the mean square among groups is $\sigma^2 + \frac{n}{1-a}\sum^{a}\alpha^2$, where a = the number of items in a group and n = the total number in the analysis. The significance of the term containing α^2 is tested by dividing the mean square among groups by the error mean square to obtain the value of F_s $(1 + f[\sum^{a}\alpha^2])$ and consulting statistical tables for the probability of this value occurring with the appropriate degrees of freedom. This analysis was performed using the computer programme ANOVA2 in APL Library 2. When only two groups are present, this analysis is equivalent to the unpaired Student's t-test, with $t^2 = F_s$. The equation for t is:

$$t = \frac{(\bar{Y}_1 - \bar{Y}_2) - (\mu_1 - \mu_2)}{\sqrt{\frac{2}{n}\sigma^2}}$$

where $\bar{Y}_1$ and $\bar{Y}_2$ are estimates of the means of groups 1 and 2 (μ_1 and μ_2), respectively.

Since, by the Null hypothesis, $\mu_1 = \mu_2$:

$$\bar{Y}_1 - \bar{Y}_2 = t_{\alpha[\nu]}\sqrt{\frac{2}{n} MS_{error}}$$

Substituting the value of t at a significance level of α and ν degrees of freedom,

$$\bar{Y}_1 - \bar{Y}_2 = \text{Least significant difference.}$$

If the test is one-tailed and t is substituted appropriately,

$$\bar{Y}_1 - \bar{Y}_2 = \text{Least significant increase (or decrease).}$$

This value is useful for illustration of the differences between treatment groups, although not all comparisons are mathematically valid.

The two-way analysis of variance reported in Table 20 was a mixed model, one factor being fixed and due to treatments, while the other factor (animals) was random. There were six replications in each group. The equation for this analysis is:

$$Y_{ijk} = \mu + \alpha_i + B_j + \gamma_{ij} + \varepsilon_{ijk}$$

where α_i is the fixed treatment effect for the ith treatment group

B_j is the added variance component due to the jth animal

γ_{ij} is the interaction between the ith treatment and the jth animal

ε_{ijk} is normally distributed, with mean = 0 and variance = σ^2.

The expected mean squares are:

Treatments:	$\sigma^2 + n\sigma^2_{A\times B} + \frac{nb}{a-1}\Sigma\alpha^2$
Animals:	$\sigma^2 + na\sigma^2_B$
Interaction:	$\sigma^2 + n\sigma^2_{A\times B}$
Error:	σ^2

where a = number of items per treatment group and b = number of items

per animal. The F_s value for added variance due to animals $(1 + f[\sigma^2_B])$ is obtained by dividing the appropriate mean square by the error mean square, while the F_s value for treatments is the quotient of treatment mean square and interaction mean square. This analysis was performed using the method described in 'Biometry' by R.R. Sokal & F.J. Rohlf (1969), Freeman Press. When only two treatment groups are present, this analysis reduces to the paired *t*-test.

The complete block design of two-way analysis with replication was performed using the computer programme ANOVA in APL Library 2. This is a programme for 3-way analysis of variance, with a facility for pooling all interaction terms involving a random factor if the data are entered appropriately. In the situation where an interaction between drugs is sought, with replication from different animals, the apparent interaction terms between animals and drug A, animals and drug B, and animals and A×B interaction may be considered to reflect the error inherent in the biological system. The equation for the analysis was therefore:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_{ij} + D_k + \varepsilon_{ijkl}$$

where α_i is the fixed treatment effect of drug A at the ith level

β_j is the fixed treatment effect of drug B at the jth level

γ_{ij} is the interaction between treatments A and B

D_k is the added variance due to the kth animal

ε_{ijkl} is normally distributed, with mean = 0 and variance = σ^2.

The expected mean squares are:

Drug A: $\sigma^2 + nb\sigma^2_{AC} + \frac{nbc}{a-1}\sum^{a}\alpha^2$

Drug B: $\sigma^2 + na\sigma^2_{BC} + \frac{nac}{b-1}\sum^{b}\beta^2$

Animals $\sigma^2 + nab\sigma^2_{C}$

A×B Interaction $\sigma^2 + n\sigma^2_{ABC} + \frac{nc}{(a-1)(b-1)}\sum^{ab}\gamma^2$

Error $\sigma^2 + \frac{n[(a-1)b\sigma^2_{AC} + (b-1)a\sigma^2_{BC} + (a-1)(b-1)\sigma^2_{ABC}]}{(ab - 1)}$

This error mean square is used for testing all main effects and the A×B interaction.